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Title of Dissertation: "The Importance of TLR2 and Macrophages in Modulating a Humoral Response after Encountering Streptococcus Pneumoniae"

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Abstract

Title of Dissertation:

The importance of TLR2 and macrophages in modulating a humoral response after encountering *Streptococcus pneumoniae*

Sam Vasilevsky

Thesis directed by:

Clifford M. Snapper, M.D.

Professor, Department of Pathology

Streptococcus pneumoniae (Pn) is an important pathogen in many community-acquired infections, including acute bacterial sinusitis, community-acquired pneumonia, as well as in more invasive infections, such as meningitis and bacteremia. Toll-like receptors (TLRs) are a family of 12 transmembrane proteins that recognize pathogens and are expressed on various immune cells including macrophages (MΦ), dendritic cells (DC), T and B, cells and they play an important role in the initial recognition of pathogens by binding conserved moieties known as pathogen associated molecular patterns (PAMPs). Pn is known to contain ligands for TLR2, TLR4, TLR7/8, and TLR9 that can act collectively to initiate innate immunity. This is largely mediated through phagocytosis and intracellular killing by neutrophils and macrophages that are

recruited to, and activated at, the site of infection (1). Adaptive immunity is initiated when professional antigen-presenting cells (APCs), such as macrophages (MΦ) and dendritic cells (DC) recognize microbial surface components via cell surface receptors (i.e. TLRs and scavenger receptors) phagocytose the bacterium, produce numerous cytokines/chemokines and migrate to secondary lymphoid organs such as the spleen and lymph nodes. Once in the secondary lymphoid tissue, the APCs activate T cells leading to many immune effector mechanisms, including the production of antibodies by B cells

In the following dissertation, I have used a murine model to study the host response to Pn. Specifically, I have focused on the ability of macrophages to induce a primary humoral response after encountering Pn and on previous findings in the lab showing a reduction of T_H1- associated (IgG2a, IgG2b, IgG3) but not T_H2-associated (IgG1) antibodies in $TLR2^{-/-}$ mice that were challenged with Pn. I found that TLR2 on B and T cells but not on DC plays a critical role in modulating a type 1humoral response when challenged with Pn. In addition, I have found that MΦ pulsed with intact *S. pneumoniae* play an active role as APCs in eliciting an anti-protein response upon adoptive transfer into naïve mice, despite the prediction that only DC could mediate this function. The protein response required viable MΦ, was T-dependent and required surface expression of CD40 and MHC- II.

The importance of TLR2 and macrophages in modulating a humoral response after encountering *Streptococcus pneumoniae*

by

Sam Vasilevsky

Dissertation submitted to the Faculty of the Molecular and Cell Biology Interdisciplinary Graduate Program of the

Uniformed Services University of the Health Sciences

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To my family, the most wonderful people I know. You will never know how much I appreciate your love, guidance and support. Thank you from the bottom of my heart for always being there for me. Mom, Dad I dedicate this dissertation to you. You are without a doubt the two bravest people I have ever known.

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Abbreviations

Ab-Antibody
αδ-dex-Dextran-conjugated anti-IgD
Ag-antigen
AP-Alkaline phospatase
APC-Antigen presenting cell

BCR-B cell receptor
BM-Bone marrow
BMDC-Bone marrow derived dendritic cell
BMM-Bone marrow derived macrophage

CFU-Colony forming units
CD40L-CD40 ligand
CTL-Cytotoxic T lymphocytes

DC-Dendritic cell
DT-Diphtheria toxin

ELISA-Enzyme-linked immunosorbent assay
EMC-Encephalomyocarditis

FACS-Fluorescence-activated cell sorting

GM-CSF-Granulocyte macrophage colony stimulating factor

IFN-γ–Interferon gamma
Ig-Immunoglobulin
IL-6-Interleukin 6
IL-12-Interleukin 12
IL-10- Interleukin 10
i.p.-Intraperitoneal
i.v.-Intravenous

KLH-keyhole limpet hemocyanin

LCM-Lymphocytic choriomeningitis virus
LPS-lipopolysaccharide
LTA-Lipoteichoic acid

mAb-monoclonal antibody
M-CSF-Macrophage colony stimulating factor
MFI-Mean fluorescence intensity

MΦ–Macrophage
MHC-II-Major histocompatibility complex class II
MHC-I-Major histocompatibility complex class I
Mtb-Mycobacterium tuberculosis
MyD88-Myeloid differentiation factor 88

NF-κB-Nuclear factor kappa B

PAMPs-Pathogen associated molecular patterns
PerM-Peritoneal macrophage
PC-phosphorylcholine
PGN-Peptidoglycan
Pn-*Streptococcus pneumoniae*
Pn14-*Streptococcus pneumoniae* serotype 14
PnP-Pn14-derived protein extract
PRRs-Pattern recognition receptors
PS-polysaccharide
PsaA-Pneumococcal surface adhesin A
PspA-Pneumococcal surface protein A
PPS14-Capsular polysaccharide serotype 14

RBC-Red blood cells
RT-Room temperature

s.c.-Subcutaneous
SN-Supernatant

TCR-T cell receptor
TD- T cell dependent
TGF-β–Transforming growth factor β
TI- T cell independent
TIR-Toll/interleukin-1 receptor
TLR-Toll-like receptors
TLR2-Toll-like receptor 2
TNF-α–Tumor necrosis factor alpha
TRAF6-Tumor necrosis factor receptor-associated factor 6
Treg-T regulatory cells
[^{3}H]-TdR-Tritiated thymidine deoxyriboside

WT-wild type

Table of Contents

Table of Figures

Chapter One

Introduction

Preface

Pn is an important pathogen in many community-acquired respiratory infections, including acute bacterial sinusitis, acute otitis media, community-acquired pneumonia, as well as in more invasive infections, such as meningitis and bacteremia. Although two vaccines are available to prevent invasive disease, they either do not cover all the current serotypes in circulation, or do not offer protection in certain age groups.

This dissertation will focus on the host response to *S. pneumoniae*. Specifically, I have investigated the ability of MΦ to elicit a humoral response in a naïve animal and the role that TLR2 (Toll-like receptor 2) plays on cells critical for a type 1 humoral response after encountering Pn. The introduction to this dissertation will provide background information that I believe will be helpful in better understanding my work and will be broken down into a number of sections. Because I have used *Streptococococcus pneumoniae* type 14 as a model for the humoral response of a host to an an intact bacterium an overview of the organism will first be presented. This will include 1) pathogenesis of the organism; 2) structure and virulence factors, specifically focusing on the capsular polysaccharide of Streptococcus type 14 (PPS14), the phosphorylcholine determinant of the teichoic acid (PC) and a surface protein, pnuemococcal surface protein A (PspA). The reason for focusing on these specific moieties is because in this thesis I will be showing data for IgM and IgG responses specific for these antigens; 3) the mechanism by which the immune system clears the bacterium including cells (MΦ, DC, CD4+T cells, and B cells) and cytokines (IL-6, IL-12, TNF-α, IFN-γ) that are important in this process.

The first part of the thesis will focus on the importance of Toll-like receptor 2 (TLR2) in modulating a type I-associated humoral response after immunization with *Streptococcus pneumoniae* type 14. Therefore, in the introduction section I have provided background information on Toll-like receptors. The second part of my thesis investigates the ability of DC and MΦ acting as APC to initiate a humoral response after encountering *Streptococcus pneumoniae*. Data will be presented on the ability of in vitro Pn14 pulsed MΦ and DC to 1) secrete IL-6, IL-12, TNF-α, IFN-γ 2) induce a protein and polysaccharide specific humoral response after transfer into a naïve animal. Hence, I have included sections describing APC, MΦ, DC important proinflammatory cytokines (IL-6, IL-12, TNF-α, INF-γ) and an explanation of how these cells and mediators are important in modulating adaptive immunity. In addition since humoral immunity involves T cells, B cells and antibodies, I have provided background on naïve T cells, effector T cells, B cell development, B cell subsets, B cell activation and antibody molecules. After the introduction, a brief explanation of the model used in the lab is discussed and the hypothesis and specific aims of this dissertation are presented.

Streptococcus pneumoniae background, colonization , invasion and pathogenesis

Streptococcus pneumoniae (Pn), also referred to as pneumococcus, is a gram-positive anaerobic bacterium and an important cause of many community-acquired infections including pneumonia, meningitis and bacteremia (2). Pneumococcus, originally named *Diplococcus pneumoniae* and renamed *Streptococcus pneumoniae* in 1974, was first isolated in 1881 by Louis Pasteur and independently by George Sternberg (3). The nasopharynx is the most common site of *S. pneumoniae* colonization and most individuals live with pneumococcus without any symptoms (4, 5). The immune system usually prevents the colonization of pneumococcus to progress into disease. However, if the immune system is compromised by defects in cough reflexes, mucosal secretion, ciliatory transport (6, 7) or the mucosal barriers of the nasopharynx are breached by the secretion of hydrogen peroxide by *S. pneumoniae* (8), then pneumococcus can travel to various parts of the body including the lungs and blood and cause pneumonia or septicemia respectively (9). If pneumococcus reaches the blood, it can then travel to the subarchnoid space of the brain breaching the blood brain barrier by damaging the tissues (10) or by transportation within infected leukocytes (11-13) thereby causing meningitis. In addition, the virulence of the strain may be a factor in whether an individual becomes ill with *S. pneumoniae* because most individuals become infected after acquiring new serotypes (14, 15).

Streptococcus pneumoniae is pathogenic mainly because it elicits a strong continuous inflammatory response that subsequently causes tissue damage. The first step occurs when pnuemococci die and their cell walls lyse and disintegrate, thereby attracting leukocytes to the site of infection. In fact, bacterial components such as the cell wall are

much more effective chemoattractants than the intact bacterium. The way leukocytes such as MΦ and DCs recognize the bacterium as foreign is through conserved moieties on the bacterium surface called PAMPs (pathogen-associated molecular patterns). After recognition of the bacterium has occurred, the leukocytes are activated to secrete pro-inflammatory cytokines and chemokines, attracting more phagocytes to the site of infection. Simultaneously, activated antigen presenting cells migrate to secondary lymphoid organs where they initiate a humoral response to the bacterium by activating T cells and by stimulating B cells to secrete antibodies specific for moieties present on the bacterial surface.

Structure of Pn and virulence factors

The main components of *Streptococcus pneumoniae* are: 1) the cell wall, which is mostly composed of peptidoglycan and binds the capsular polysaccharide, the cell wall polysaccharide and various surface expressed proteins including the important virulence determinant PspA; 2) a cell wall; 3) and a polysaccharide capsule (16). Another important component of the pneumococcus is choline. This compound is an essential for the growth of the bacterium (17-19) and binds a number of proteins termed choline binding proteins (CBPs) that are expressed on the surface of the pneumococcus (20, 21). Choline in *S. pneumoniae* mostly occurs as chains of teichoic acids (TA) or lipoteichoic acids (LTA) (teichoic acids with a lipid component) (20, 21) and when attached to phosporyl groups are called phosporylcholine. In *S. pneumoniae* phosyphorylcholine is used as a linker to bind LTA with choline binding proteins. Both phosphorylcholine and the CBP PspA are

considered PAMPs and antibodies to these conserved moieties have been shown to be protective against pneumococcus (22, 23).

The choline binding protein PspA is found on every single serotype of *S. pneumoniae* (24), is required for full virulence, and can range between 76 to 99 kDa depending on the strain (25, 26). The reason PspA is termed a virulence factor is because it prevents the attachment and activation of complement on the pneumococcus surface (27, 28). PspA is a highly charged and polar protein that helps to stabilize the electronegative capsule with a positive domain and repels complement with its external negative domain (29, 30). Because of its ability to resist phagocytosis, the pneumocccal polysaccharide capsule is considered a key virulence factor (16). In addition, the nomenclature regarding different strains of *S. pneumonie* is based on variations of the polysaccharide capsule. There are currently 90 strains, also referred to as serotypes, of *S. pneumoniae.*

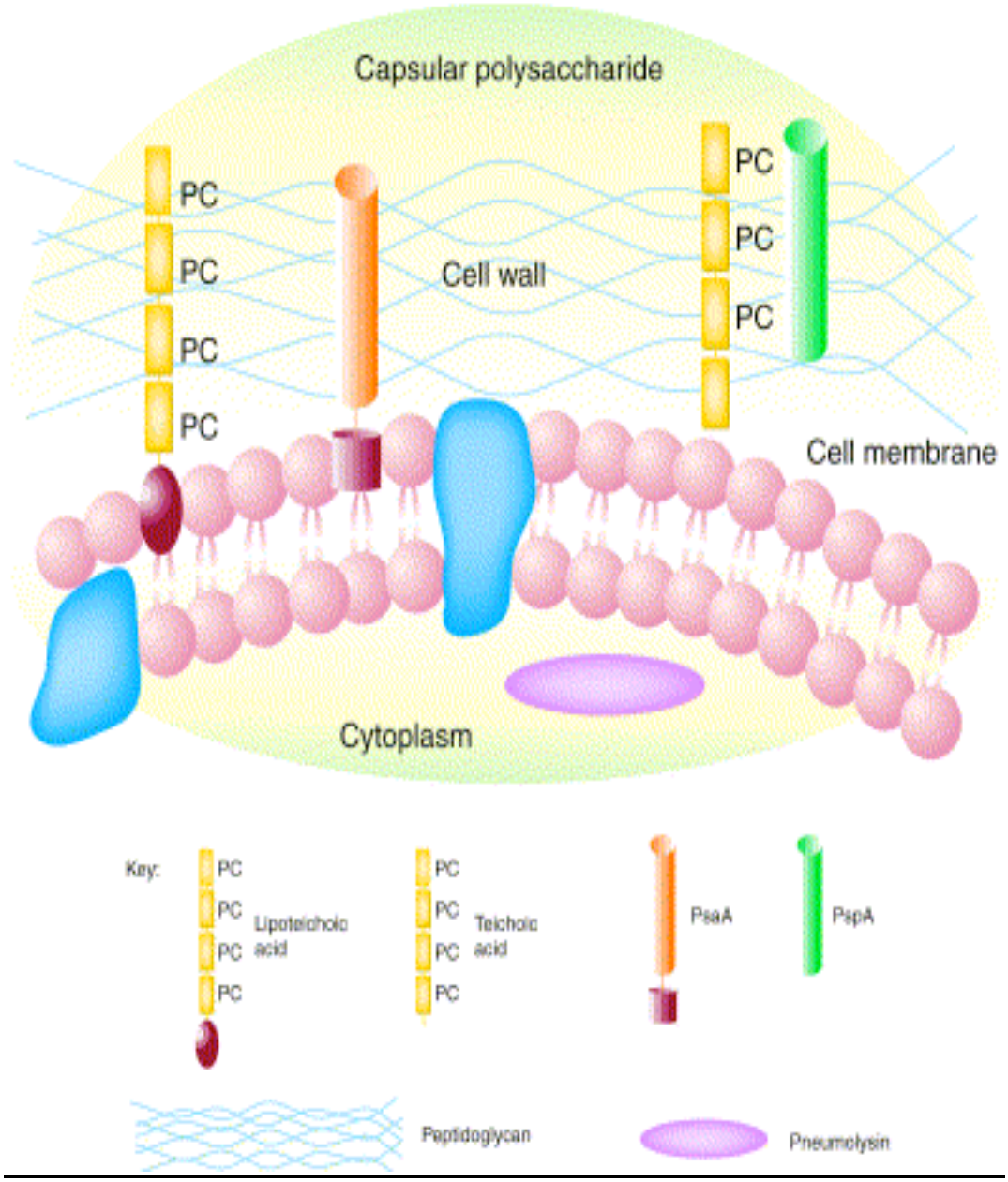

Capsular polysaccharide
PC
PC
PC
PC
Cell wall
PC
PC
PC
Cell membrane
Cytoplasm
Key:
PC
PC
PC
PC
Lipoteichoic acid
PC
PC
PC
PC
Teichoic acid
PsaA
PspA
Peptidoglycan
Pneumolysin

Figure 1. *Streptococcus pneumoniae* virulence factors

The outer layer of *S. pneumoniae* is composed of capsular polysaccharide, which is attached to the bacterium by chains of teichoic acid, and the cell wall, which is comprised of peptidoglycan. Although the capsular polysaccharide acts as an outer barrier by enveloping the bacteria, its lattice like structure enables receptors on immune cells to have access to epitopes inside the capsule such as PC and PspA. PspA is a surface protein that is considered a pneumococcal virulence determinant. It interferes with complement activation thereby preventing complement-mediated opsonization. PC is another important epitope on the bacterial surface that acts as an adhesive factor to allow initial attachment to the host cell.

Protection against Pn

Phagocytic leukocytes (i.e. MΦ and PMN) are critical for the clearance of pneumococcal infection because they bind, phagocytose and kill the bacterium in their phagolysosomes (31). However, since most of the virulent strains of *S. pneumoniae* contain a polysaccharide capsule that hinders phagocytosis (32), complement and antibodies that bind to specific moieties on the pneumococcus, are extremely important in the effective clearance of this bacterium . In this regard pneumococci that are opsonized with compliment and / or antibodies are easier to phagocytose than non-opsinized bacterium (33). Complement, which is one of the first lines of defense against a pathogen and considered a component of the innate immune system, can either bind to antigen- antibody complexes on the bacterial surface or it can it can directly bind to *S. pneumoniae* and lyse the bacterium (34, 35). Even more critical for the clearance of pneumoccus are antibodies. Antibodies are produced by B cells and are a key component of the humoral response to pneumococcus. In addition to acting as a bacterial opsonin antibodies are also able to block ligands on the pneumococus that help it to attach itself to epithelial cell receptors.

The distribution of antibody isotypes produced in response to a pneumococcal infection is important for the clearance of the bacterium. Particularly critical are the T_H1-associated gamma interferon (INF-γ)-dominant IgG isotypes, commonly referred to as type 1 antibodies. These include IgG2a, IgG2b, and IgG3, which are able to both bind to bacterial surface epitopes and fix complement thereby mediating bacterial opsonophagocytosis (36). In contrast IgG1, which is a T_H2-associated interleukin 4 (IL-

4)-dominant IgG isotype, is not considered a good activator of compliment or inducer of inflammation (37).

Cytokines and cells important for the clearance of Pn

Cytokines released early post-infection by MΦ and DC are important not only in mediating an innate immune response but are also critical for the induction of humoral immunity to *S. pneumoniae (38)* . These cytokines include but are not limited to IL-6, IL-12, TNF-α and IFN-γ. TNF-α helps to increase the amount of IgG and complement in the tissues by mediating vascular permeability. Even more importantly for the humoral response, TNF-α helps to skew T cells to secrete INF-γ by costimulating APC to produce IL-6 and IL-12 and thereby inducing a type 1 antibody response. IL-12 also plays a role in antibody production. Not only does IL-12 affect class switching by stimulating the production of INF-γ by CD4+ T cells (38-40) but it also can also stimulate Ig secretion by postswitched B cells in an Ig isotype-nonselective and IFN-γ-independent manner (41). IL-6 is important in that it may stimulate the synthesis of the complement component C3 by macrophages and through direct induction of B cell maturation (42)

Antigen-presenting cells (APC) such as dendritic cells (DC) and MΦ are key components in the response to bacteria because they secrete IL-6, IL-12, TNF-α, and IFN-γ and act a bridge between innate and adaptive immunity by activating T cells thereby inducing a humoral response. APCs phagocytose Ag via their cell surface receptors and attach the peptides onto the major histocompatibility complex (MHC) class I and II pathways for presentation to T cells. Presentation of MHC-II bound peptide on the APC to the CD4+ T cell, APC expression of co-stimulatory molecules such as CD80,

CD86, and CD40, and cytokine secretion induces the T cell to differentiate into one of a number of distinct T cell subsets (e.g. T_H1, T_H2, T_H17, or Treg). In fact a direct link has been reported indicating that the secretion of IL-6 by DC is critical for the induction of humoral response to *Streptococcus pneumoniae*. Colino *et al.* observed that DCs obtained from IL-$6^{-/-}$ mice are defective, relative to wild-type (wt) DCs, in eliciting in vivo Ig responses when pulsed in vitro with Pn and adoptively transferred into naïve mice (43)

T cell-independent (TI) vs T cell-dependent (TD) antigens

There are two general types of antigens commonly referred to as either T cell-dependent (TD) or T cell-independent (TI). TI antigens can be further subdivided into TI type 2 or TI type 1. The most common TI-2 antigens are polysaccharides and are characterized by fact they contain repeating epitopes that are able to multivalently attach to receptors on immune cells. Additionally, TI-2 Ag have large molecular weight, are able to activate complement but unable to activate an MHC II- dependent T cell response (in which cognate interactions between antigen presenting cells and CD4+ T cells are required because of the there inability to bind to MHC class II molecules (44, 45)). Because polysaccharide molecules express identical repeating epitopes that can bind multivalently to B cell receptors and are unable to bind to MHC class II molecules, it is thought that these antigens are able to directly activate B cells along with noncognate T cell help (46). However considerable evidence has accumulated indicating that T cells, acting in a non-cognate manner may, under certain circumstances, play a significant role in regulating specific antibody responses to bacterial polysaccharides (43, 45, 47-50). TI-

1 antigens, on the other hand, are usually polysaccharides attached to a TLR ligand and are mitogenic to B cells, with lipopolysaccharide (LPS) being a common example of this type of antigen (45). Unlike the two types of TI antigens , TD antigens are presented by APC to CD4+T via MHC class II molecules thereby inducing a T dependent response and subsequent memory if reinfection occurs (16) .

Toll-like Receptors (TLRs) and TLR2

Immune cells such as DC, MΦ, B cells and T cells recognize specific evolutionary conserved moieties on pathogens called PAMPs (pathogen-associated molecular patterns) via germline-encoded receptors commonly referred to as pattern recognition receptors (PRRs). Examples of PAMPs include LPS, peptidoglycan and double stranded DNA. PRRs can either be intracellular, secreted or expressed on the surface of the leukocyte (51). An important group of PRRs are the Toll-like receptors (TLRs).

Toll receptors were originally discovered in Drosophila as type I transmembrane proteins that play an important role in embryonic development and antifungal immunity (52, 53). A mammalian homolog of the toll receptor was first identified in 1985 and was termed Toll-like receptor 1 (TLR1). Currently there are 12 known Toll-like receptors, which have been found to be expressed on DC, MF, T cells, B cells, vascular endothelial cells, adipocytes, cardiac myocytes and intestinal epithelial cells (54, 55). TLRs 1, 2, 4, 5 and 6 are expressed on the surface and seem to be important in the recognition of moieties unique to bacteria, whereas TLRs 3, 7, and 9 are sequestered intracellularly in late endosomes-lysosomes and specialize in viral detection by recognizing nucleic acids

(56-60). Since pathogens express ligands for several TLRs, these ligands often cooperate to activate multiple TLRs during infection (61-66).

TLR2 is an important receptor in recognizing important moieties on gram-positive bacteria including peptidoglycan, lipoproteins and lipoteichoic acid (67-71). Originally TLR2 was thought to be an important receptor recognizing LPS (72, 73). However further research showed that cells lacking TLR2 responded normally to LPS (74) and that a lipoprotein contaminant in the LPS preparation was the real TLR2 ligand (67). Interestingly, recent evidence has suggested that some TLR2 may be able to recognize some type of LPS (e.g. Porphyromnas gingivalis) (75).

Most TLR signaling occurs through an important adaptor protein called MyD88 (myeloid differentiation factor 88), although MyD88 -independent signaling can occur as well (54, 76, 77). TLR3 is the only TLR that is completely MyD88 independent, signaling through the adaptor protein TRIF, also known as TICAM (78, 79). In addition TLR4 can utilize both a MyD88-dependent and a MyD88-independent pathway that utilizes both TRIF and another adaptor protein called TRAM (80, 81). In the MyD88-dependent pathway, MyD88 interacts with IRAK4 and IRAK1 in the cytoplasm via homophilic death domain interactions. Activated IRAK 4 phosporylates and activates IRAK1 which subsequently interacts with TNFR-associated factor-6 (TRAF6) (82), causing the oligomeration and activation of TRAF6 (83-86). TRAF6 activates TAK1 kinase leading to the activation of IKK kinase complex. IKK kinase phosporylates IκB resulting in its ubiquitination and subsequent proteosome-mediated degradation causing the release of the trascription factor NF-κB into the nucleus and the transcription and production of various chemokines and cytokines (86-88).

NF-κB designates a group of heterodimeric transcription factors that share common characteristics in their structure and function. Currently there are five known mammalian NF-κB proteins, including c-Rel, RelA (p65), RelB, NF-κB1 (p50 and its precusor p105), and NF-κB2 (p52 and its precusor p100) (89, 90). All members NF-κB proteins, except for p50, are able to both homo and hetero dimerize with each other with the p50/65 and p52/65 being the most common heterodimers (89).

Professional APC

Professional antigen presenting cells (e.g. DC, B cells, and MΦ) are critical for an adaptive immune response because they are able to present antigenic peptides to CD4+ T helper cells initiating a humoral and/or cell-mediated immune response. Sentinel APCs such as MΦ and DC constantly sample antigens in areas such as tissues, blood or lymph fluids. APCs encountering pathogens such as *S. pneumoniae* will phagocytose the bacterium, and migrate to secondary lymphoid organs (91) where CD4+ T cells can be activated via APC MHC class II molecules along with important costimulatory molecules such as CD80 (B7-1), CD86 (B7-2) and CD40 (92). These activated T cells can in turn induce B cells to proliferate and secrete antibodies resulting in a humoral immune response to the bacterium.

Antigen processing and presentation

T cells are only able to recognize Ag when it is bound to an MHC molecule. CD8+ T cells recognize cytosolic Ag that have been processed, degraded and presented

on MHC class I molecules and hence are important in cancer and viral infections (93). CD4+ T cells, on the other hand, recognize exogenous proteins that have been ingested through endocytosis, pinocytosis or phagocytosis (94-96) and presented on MHC class II molecules. Antigenic peptides vary in size depending on whether they are attached to MHC class I or MHC class II. Peptides bound by MHC class II tend to be longer with peptides at least 13 amino acids (97-99) whereas peptides ranging from 8-11 amino acids are typical for MHC class I (100, 101). Only professional antigen presenting cells such as B cells, DC, and MΦ have the ability to express MHC class II while nearly all nucleated cells express MHC class I.

The MHC class II molecule is a heterodimer that includes a groove where peptides are able to bind (102, 103) and is assembled in the endoplasmic reticulum (ER). The MHC class II molecule exits in the ER with the help of a protein called the MHC class II-associated invariant chain (Ii) (104, 105). Once the MHC class II molecule leaves the ER the invariant chain is cleaved by cytosine and aspartic proteases leaving a peptide fragment called CLIP (Class-II-associated invariant chain peptide) in the peptide-binding groove. CLIP sits in the antigen-binding groove in a specialized edosomal/lysosomal compartment termed MHC class II compartment (MIIC) until it is ready replaced by peptides (96, 106, 107). The peptide bound MHC class II molecule then travels to the surface via the default exocytosis pathway.

Dendritic Cells

DCs are a heterogeneous population of leukocytes that arise from the bone marrow and are considered an important link between innate and adaptive immunity. DC

are considered one of the main sentinel immune cells and reside in most tissues in the body. Immature DC are extremely phagocytic and scour the environment for potentially harmful invaders whether it be pathogenic bacteria, viruses or toxins (108). In this immature state they express low levels of MHC class II and the costimulatory molecules CD80 and CD86. However, after recognition and acquisition of Ag, DC begin their maturation process by secreting cytokines and chemokines, migrating to secondary lymphoid organs and initiating a potential adaptive immune response (108-111). Once the DC have matured they lose their ability to phagocytose but express high levels of MHC class II (109, 110), CD80 and CD86 molecules (112-114). In this mature state DC are found in the T cell zones of the secondary lymphoid organs where they are able to interact with, and activate naïve T cells via MHC-II, CD80, CD86 and CD40.

The 3 major subsets of DC are referred to as plasmacytoid, myeloid and lymphoid. Plasmacytoid DC are regarded as a major producer of type 1 interferons (e.g. IFN-α and IFN-β) and are $CD11c^{+}CD11b^{-}B220^{+}$ whereas both lymphoid and myeloid DC do not express B220. There are several factors that distinguish lymphoid from myeloid DC. Myeloid are more phagocytic than lymphoid DC; lymphoid DC express the CD8α homodimer while myeloid DC are $CD8^{-}CD4^{+}$; lymphoid DC produce higher levels of IL-12 upon stimulation than myeloid DC.

Macrophages

Macrophages originate from monocytes that leave the bone marrow and enter the tissues via the blood stream. The monocyte begins as a hematopoietic stem cell that divides several times at which stage it is called a monoblast (115). The monoblast in turn

divides into a promonocyte and finally into a monocyte. Like DC, MΦ are a heterogeneous population with their functional capabilities depending on the state of activation or differentiation and the site of residence (116-118). Virtually every tissue and organ contains MΦ including skin (Langerhans cells), bone (osteoclasts), liver (Kupfer cells), and brain (microglia). One of the most diverse populations of macrophages reside in the secondary lymphoid organs such as the spleen and lymph nodes. In the spleen macrophages can differentiate into metallophilic macrophages, white pulp macrophages, marginal zone macrophages, and red pulp macrophages (119-121). Macrophages, in contrast to DCs, typically express lower levels of MHC-II, CD80, CD86 and CD40 which contributes to their lower efficiency at priming naïve CD4+ T cells. Although macrophages are highly phagocytic, making them a good cell type to acquire foreign antigens and present them to T cells, they are highly degradative and hence express lower levels of peptide on their MHC-II than DC. This is also believed to contribute to their lower APC potential.

Priming of naïve T cells

In order for a naïve T cell to be primed interaction with an APC needs to occur. Activated APC that have processed and presented antigen on their MHC molecules migrate to the spleen or lymph nodes and present the peptide-MHC complex to the T cell receptor (122, 123). However if costimulation does not occur such as the critical interaction between CD80 (B7.1) and CD86 (B7.2) on the APC with its receptor CD28 on the T cells (123-126), then the T cell will become anergic or nonresponsive (124-126).

Additionally cytokines produced by the APC have been shown to be important contributors for T cell activation and differentiation (127). CD40-CD40L interaction is another important costimulatory signal for both T cell and APC activation. CD40L is upregulated on the T cell by antigen-driven interaction with APC and binds CD40 on the APC, which leads to optimal activation of APC costimulatory molecules.

T cell development

T lymphocytes, just like all hematopoietic cells, are derived from the bone marrow. However, whereas the majority of hematopoietic lineages mature in the bone marrow, T cell development takes place in the thymus. Cells that are destined to be T lymphocytes are double negative ($CD8^-CD4^-$) before they enter the thymus (128, 129). After entering the thymus through the cortico-medullary junction and undergoing a rigorous selection process, these T cell precursors will mature into either CD^-CD4^- γ:δ T cells or α:β (CD4+CD8- or CD4-CD8+) T lymphocytes, the latter becoming the majority of the T cell population (130). The selection process begins after the double negative T cells migrate from the cortico-medullary junctions to the subcapsular region of the thymic cortex. In the subcaspular region, the $CD4^-CD8^-$ cells develop into double positive thymocytes (CD4+CD8+) that express low levels of the TCRαβ antigen-receptor complex (131). Thymocytes then undergo a complex process of positive and negative selection, that consists of enriching double positive T cells whose receptors bind weakly to self-MHC molecules presented by APC, and which ultimately allows the survival of those $CD4^+CD8^+$ T cells that are activated by foreign but not self Ag. This process also enables the correct TCR to pair with the appropriate CD4 or CD8 co-receptor. For

example, TCRs specific for MHC II need to retain CD4, and lose CD8, whereas T cells specific for MHC class I need to retain CD8 and lose CD4 (132, 133). Additionally, if double positive T cells react too strongly to self Ag presented by APC via either MHC class I or II molecules then they will be eliminated by negative selection which occurs in the medulla (134). Another way double positive cells are eliminated is through death by neglect. This process occurs when there is too little signaling and the cells are induced to commit suicide through apoptosis (135). The cells that remain after positive and negative selections are either MHC class I restricted (CD8+) or MHC class II restricted T cells. CD8+ T cells are known to be important in viral and cancer (i.e., cellular) immunity, whereas CD4+ T cells regulate humoral immunity. Regulatory T cells are also produced in the thymic medulla, although the process of differentiation and selection of regulatory T cells is not as well understood (136). The mature single positive T cells that remain after this rigorous ordeal arrive in the periphery (i.e. lymphatic and circulatory systems) by exiting the thymus through the perivascular space (136)

Effector T cells

A naïve T cell, after encountering its specific antigen in the context of Ag-MHC complex, will proliferate and differentiate into an effector cell. The T cell is now considered primed, and if restimulated by an APC, will require a lower threshold for restimulation. There are several factors governing the signal quality for initial T cell activation including the length of APC-T cell attachment, the level of costimulation and concentration of Ag presented by the APC. For example, it has been demonstrated that a low level signal can induce a T cell to proliferate and migrate to secondary lymphoid

organs whereas a modest stimulatory signal can induce upregulation of adhesion molecules needed for tissue migration, differentiation into T_H1 or T_H2 subsets and cytokine production (137). If the APC is laden with high concentrations of Ag and there is a high level of stimulation then T cell proliferation can occur in as little as 6 hours whereas a low concentration of Ag combined with a weak stimulatory signal can take as long as 40 hours (137, 138).

Ag presented on the MHC class I molecules activate CD8+ T effector cells whereas Ag- MHC class II molecules generate effector CD4+ T cells. Effector CD4+ cells can be broken down into 5 subsets T_H0, T_H1, T_H2, T_H17, and Tregs. T_H0 cells are thought to be the least differentiated and secrete predominantly interleukin-2 (IL-2), and granulocyte- macrophage colony stimulating factor (GM-CSF). T_H1 cells are able to secrete IFN-γ, which is important in activating MΦ to produce mediators important for microbial killing such as various cytokines and oxygen intermediates. These T cells can also play a key role in the elimination of intracellular pathogens, and delayed type hypersensitivity responses via their ability to secrete IL-2 and TNF-α. T_H2 cells secrete IL-4, IL-5, IL-9, IL-10 and IL-13 (139, 140). These cytokines are important for B cell activation and isotype switching as well as allergic responses (141-144). Various pathogens require different effector CD4+ T for effective clearance. For example intercellular parasites such as Leishmania donovani require a T_H1 response (145) while helminth infections like *Schistosoma mansoni*, requires a T_H2 response (146)

Regulatory T cells, which can be subdivided into Tregs, Tr1 or T_H3 cells, are another important subpopulation of effector CD4+ T cells. Tregs, which are CD4+CD25+, mature in the thymus, are self reactive, can secrete IL-10, transforming

factor-β (TGF-β) and IL-4, and are important in the maintenance of tolerance suppression of immune responses. Both Tr1 and Tr3 mature outside the thymus after antigen stimulation and are able to secrete TGF-β with Tr3 producing large quantities of this cytokine. Tr1 cells can secrete IL-10, IL-5, and IL-13 but make little or no IL-2, IL-4 or INF-γ (147). T_H17 T cells are highly pro-inflammatory and have been shown to play a major pathogenic role in Crohn's disease (148, 149), ulcerative colitis (150), psoriasis (151), multiple sclerosis (152, 153), and experimental autoimmune encephalitis (EAE) (154)

B cell development

The bone marrow is the site of both B cell precursors and their subsequent development into mature B cells. Hematopoietic cells that are destined to become B cells first develop into pro-B cells with immunoglobulin gene assembly starting at the heavy chain loci (155). VDJ recombination occurs in the pro-B cell stages with early pro-B cells rearranging D_H-J_H genes and the late pro-B cell stage characterized by V_H-DJ_H recombination (156-158). After successful recombination in the pro-B cell stage has occurred the immature B cell them develops to the pre-B cell stage. Pre-B cells progress from large actively dividing pre-B cells where the μ heavy chain is expressed intracellularly in combination with a surrogate light chain to form the pre-B cell receptor to small non-dividing pre-B cells that cease expression of surrogate light chains and only express the μ heavy chain in the cytoplasm (159). At this point the pre-B cell develops into an immature B cell characterized by surface expression of IgM acting as a BCR and

subsequently progresses into a mature B cell that expresses both IgM and IgD on its surface (160).

B cell subsets

B cells can be generally divided into two categories, B-1 and B-2 cells. B-1 B cells are considered to be important in innate immunity, are characterized phenotypically as being $IgM^{high}IgD^{low}CD11b^{low}$, and develop before birth (i.e. fetal and peri-natal) (161, 162). B-2 cells are important for adaptive immunity and develop after birth with production continuing throughout adult life (44, 163, 164). B-1 B cells can be further subdivided by the absence or expression of the CD5 receptor. B-1 cells that are $sIgM^{hi}sIgD^{lo}CD11b^{+}CD5^{+}$ are referred to as B-1a cells whereas B-1b cells do express CD5 (162, 164). The main function of B-1 cells appears to be the production of IgM to T-independent Ag, such as phoshorylcholine in the pleural and peritoneal cavities where they comprise half of the total B cell population (161, 165). In some circumstances B-1 cells may be induced to switch into IgA secreting cells (166).

B-2 B cells can be divided into follicular B cells and marginal zone (MZ) B cells and participate in the vast majority of responses to exogenous antigens. Follicular B cells are the predominant type in the spleen and lymph nodes and a minor component of lymphocytes in peritoneal cavity and bone marrow. Although marginal zone B cells are a minor population in the spleen, mainly residing outside the marginal sinus, they do however predominate in peritoneal and pleural cavities (76, 163, 167, 168). Mature follicular B cells do not express CD5 or CD11b, but do express low levels of IgM and

high levels of B220, CD23 and IgD and are generally smaller than both B-1 and MZ B cells whereas MZ B cells are also B220 and IgM high but IgD and CD23 low (163, 167-169).

B cell activation

Generally, two signals are required for B cell activation. Ag that are either soluble, presented by APCs or bound by antibody (immune complexes) arriving to the secondary lymphoid organs (i.e. lymph nodes, spleen) are recognized by naïve B cells and bind to their membrane bound Ig. Ag arriving from the blood are filtered by the spleen, entering the white pulp through the marginal sinus (170) where they are first encountered by B cells, macrophages and DCs in the MZ (171). Small molecular size Ag are able to directly filter through to the lymphoid follicles whereas larger Ag complexes cannot diffuse passively into the follicles and require active transport (172, 173). In fact there is evidence that both DC and B cells are able to transport Ag into the B cell follicles (174) and transfer them onto follicular dendritic cells (FDC) (175). B cells that have encountered their specific Ag relocate to the B-T cell zone inside follicles (176, 177) where they receive signal number two. The second signal is provided by APC activated CD4+ T cells that have encountered the same Ag as the B cell. Proliferation and activation is induced by the binding of the TCR to the B cell antigen-loaded MHC class II, CD40 interaction with CD40L (178) and CD28 interaction with CD80 (179), along with other costimulatory molecules (180). Activated B cells can either become long-lived

plasma or memory cells (181), extrafollicular antibody-secreting plasma cells or form germinal centers (182).

Germinal centers are areas in secondary lymphoid organs (i.e. spleen, lymph nodes, Peyer's patches and tonsils) where Ag specific B cells proliferate, and undergo affinity maturation and isotype switching in order to produce high affinity antibodies. Initially, germinal centers start forming within 4-5 days after an infection. Maximum proliferation of cells occurs by 10-11 with a steady decline after three weeks (183). B cells that have been activated by Ag proliferate and differentiate into centroblasts in the dark zone of the germinal center. At this juncture a process known as somatic hypermutation occurs where immunoglobulin V(D)J genes are rearranged (184). Centroblasts subsequently differentiate into centrocytes and move into the light zone of the germinal center. This area is the site of both clonal selection and isotype switching. Clonal selection refers to the process by which B cells that have undergone somatic hypermuation and have the highest affinity for Ag are selected, with the aid of follicular dendritic cells T cells and APCs, for survival. Those B cells that have weak or no affinity for Ag are removed by apoptosis (185). The centrocytes that survive, eventually become either memory B cells or plasma cells. Isotype class switching enables B cells to switch their immunoglobulin expression from IgM and IgD to other classes of antibody. This is especially important for the removal of extracellular bacteria such as *Streptococcus pneumoniae* because it has been shown that IgG isotypes are critical for clearance of pneumococcal disease (16). Critical for isotype switching are cytokines and co-stimulatory signals including CD40-CD40L interaction (184). In addition there have been reports that ICOS (inducible T cell co-stimulator), TACI (transmembrane activator

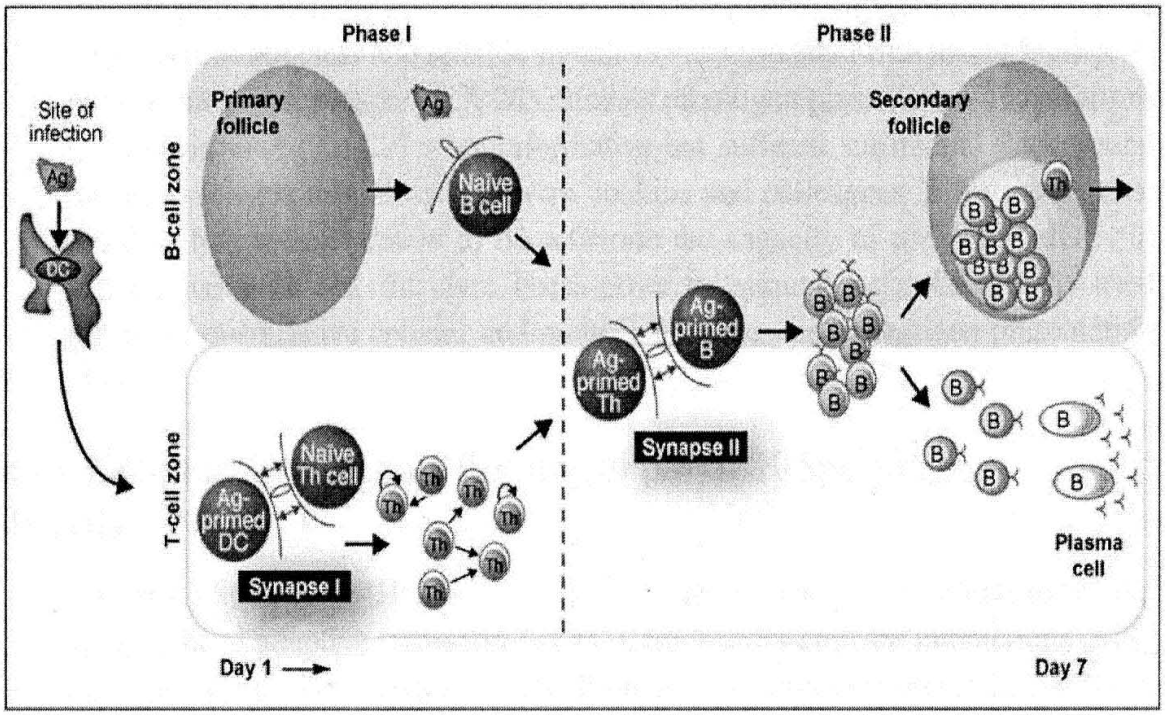
and calcium-modulating cyclophilin-ligand interactor), and BAFFR (B-cell-activating factor receptor) also play important roles in isotype switching (184, 186-188)

Antibodies

Antibodies, also known as immunoglobulins (Ig), are glycoprotein molecules produced by antibody producing B cells called plasma cells that bind to specific antigenic determinants. They have many functions including neutralization of toxins (189), immobilization of microorganisms (190), neutralization of viral activity (191), and activation of complement (192), and they are found in the blood, tissue fluids and secretions (193-195). Immunoglobulins are comprised of 2 heavy and 2 light chains with a disulfide bond linking them together and are categorized based on the differences in their heavy chain constant regions (IgM, IgG, IgA, IgD, IgE). Additionally, the IgG immunglobulins can be subdivided IgG1, IgG2, IgG3, IgG4 for humans (196) and IgG1, IgG2a, IgG2b and IgG3 for mice (197).

Immunoglobulin A (IgA) is mostly found in secretions, can exist as either a dimer or a monomer, has a characteristic T shaped structure, and can be subdivided into IgA1 and IgA2 (198, 199). Because IgA is secreted across the mucosal surfaces it is important against pathogens that reside in the stomach, intestines, and lungs. In addition is also found in maternal milk, tears and saliva and does not bind complement (200-202). IgD is important in that along with IgM it is the predominant membrane bound immunoglobulin on mature naïve B cells. Additionally, IgD exists as a monomer and just like IgA is unable to bind complement (203, 204). IgE exists as a monomer but does not bind

complement. It is important in parasitic helminth diseases and because of its ability to tightly bind and activate basophils and mast cells is an important mediator of allergic reactions (205, 206). IgM exists as monomer when it is bound to the surface of a B cell but is secreted as a pentamer or a hexamer and is the most potent antibody isotype in activation of the classical complement pathway (207-209). IgG is a monomer, an effective complement activator (210), able to neutralize toxins (211), able to prevent viruses from attaching to target cells and gain entry (212) and is important in protecting the fetus because it is the only isotype able to pass through the placenta. In addition IgG is able to immobilize bacteria (213) and is considered an opsonizing antibody (214) because of its ability to bind epitopes on microorganisms and trigger complement deposition on their surfaces, thereby facilitating phagocytosis of the pathogen by phagocytic cells.


Phase I
Phase II
Site of infection
Ag
DC
B-cell zone
Primary follicle
Ag
Naive B cell
Secondary follicle
Th
T-cell zone
Naive Th cell
Ag-primed DC
Synapse I
Ag-primed B
Ag-primed Th
Synapse II
Plasma cell
Day 1
Day 7

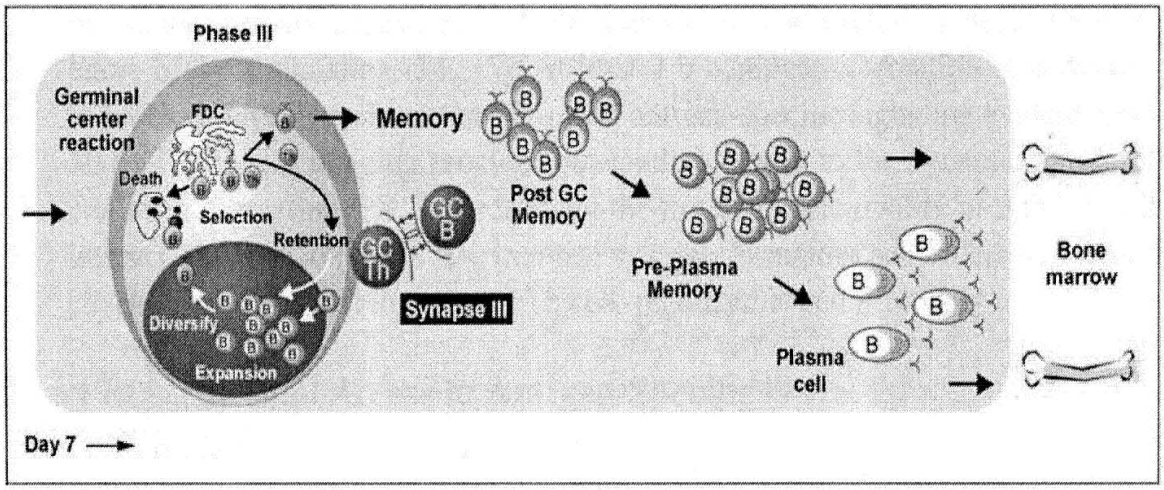

Phase III
Germinal center reaction
FDC
Death
Selection
Retention
Diversity
Expansion
Memory
GC B
GC Th
Synapse III
Post GC Memory
Pre-Plasma Memory
Plasma cell
Bone marrow
Day 7

Figure 2. An overview of the immune response to extracellular bacteria such as *Streptococcus pneumoniae*.

When an extracelluar pathogens, such as *Streptococcus pneumoniae,* invades a host, the innate response, which is non-specific and is initiated within minutes or hours, is triggered. This involves the activation of immune cells such as macrophages and dendritic cells that recognize the pathogen as foreign by virtue of conserved moieties on the surface of the pathogen known as PAMPs (pathogen associated molecular patterns). As a result, proinflammatory cytokines and chemokines are produced, which leads to the recruitment and activation of more macrophages, dendritic cells and neutraphils to the site of the infection. At this point, the adaptive arm of the immune response is initiated. APCs that have phagocytosed the pathogen migrate to secondary lymphoid organs such as the spleen and lymph nodes and present antigen in the context of MHC class II to naive CD4+ T helper cells. These T cells become activated and in turn activate B cells that recognize the same antigen. This B cell-T cell interaction allows the B cells to proliferate and develop into antibody-producing plasma cells. Antibodies are crucial to the clearance of extracellular bacteria such as *Streptococcus pneumoniae.*

Experimental Model

Streptococcus pneumoniae (Pn), capsular polysaccharide serotype 14 (Pn14), is one of the more common Pn serotypes found in carriers and reported to cause disease (215). Compared to polysaccharide- encapsulated Pn, nonencapsulated strains are essentially non-virulent (216). We use heat-killed Pn for most of our studies because it serves as a useful immunogen for the elicitation of an immune response without the possibility of killing the animal. In vivo studies were performed by administering the inoculum intraperitoneally (i.p.) versus an intranasal, intrathoracic or intrapulmonary route. For our adoptive transfer experiments, we administered the in vitro Pn-pulsed-APC (MΦ and DC) intravenously (i.v.) because that is the most direct route to the spleen.

Much of our knowledge of the regulation of the humoral immune system is based on immunization studies that use soluble, purified antigen. Much less is known about how intact pathogens induce an antibody response. Thus, using heat-killed Pn14, we are able to study responses to the major antigenic factors expressed by the intact bacteria, such as the capsular polysaccharide (PPS14), the phosphorylcholine (PC) determinant of teichoic acid, also known as the cell wall C-polysaccharide, and the cell wall protein pneumococcal surface protein A (PspA). Our lab has demonstrated that anti-protein responses are regulated differently than anti-polysaccharide responses to intact Pn in that anti-polysaccharide responses (anti-PC and anti-PPS14) occur more rapidly than the anti-protein response (anti-PspA) (217). The anti-polysaccharide titers are seen on day 4 and peak at day 6, while the anti-protein titers are first seen on day 6 and peak on day 10 (50). Additionally, only PspA-specific memory is generated. We also found that while both

anti-PC and anti-PspA responses require $CD4^+$ TCR-$\alpha\beta^+$ T cells, the anti-PC response occurs in a TCR-nonspecific manner, and in the absence of germinal center formation, which is consistent with the observed lack of PC-specific memory (218). Khan *et al.* (217) demonstrated that the IgG, but not IgM, (anti-PPS14), like IgG anti-PspA responses, are $CD4^+$ T cell dependent and TCR specific. However in contrast to the anti-PspA response, we did not observe any apparent memory, but did see accelerated kinetics of primary Ig induction and a more rapid delivery of CD4+ T cell with the anti-PPS14 response. These previous studies are relevant because in the following thesis, I will be comparing the anti-PC IgM and IgG3 responses of TLR2-/- mice relative to WT mice after immunization with *Streptococcus pneumoniae*. In addition, data will be presented comparing the ability of Pn14 pulsed- MΦ and DC to elicit anti-protein (IgM and IgG specific for PspA) and anti-polysaccharide (anti-PPS14 IgM and IgG) responses after adoptive transfer into naïve mice and the ability of MΦ and DC to induce these humoral responses in the absence of CD4+ T cells.

Because we used heat-killed bacteria for all of our experiments, we were modeling the immune response to an intact bacterium rather than any specific diseased state. However, this route of immunization stimulates a systemic, rather than, a mucosal response and may serve as a model for systemic infection, which is relevant to an organism that can cause bacteremia and subsequent sepsis. Bacteria in the peritoneum can disseminate throughout the body causing a systemic infection and when it enters the blood it can cause sepsis and septic shock. In fact *streptococcus pneumoniae* is the main pathogenic bacteria responsible for invasive diseases such as bacteremia and meningitis in children less than 2 years of age (219).

Hypothesis and Specific Aims

The general goal of this dissertation is to gain a better understanding of the innate and adaptive host immune response to Pn. Specifically, I have investigated the potential role TLR2 plays on cells of the adaptive immune system (i.e. B cells, T cells and DC) in mediating a T_H1 antibody response to Pn and the ability of MΦ to elicit a humoral response in a naïve animal in response to Pn. The hypothesis and specific aims are as follows:

Hypothesis #1: Is the expression of TLR2 on B cells, T cells and/ or DC is critical for optimal induction of a humoral response to intact *Streptococcus pneumoniae?*

We have previously demonstrated that spleen cells from TLR2-/- mice elicit a largely normal cytokine and chemokine response in vitro and in vivo in response to Pn14. This response was associated with no increase in lethality upon i.p. infection, relative to wild-type mice. In contrast, $TLR2^{-/-}$ mice exhibited a significant decrease in the elicitation of type 1 (T_H1-associated) IgG isotypes (IgG3, IgG2b and IgG2a) specific for PPS, PC and a number of Pn proteins, whereas induced serum titers of the type 2 (T_H2-associated) IgG isotype IgG1 were equivalent to that observed in WT mice (220). In light of the fact that innate immunity was largely intact, the defective type 1 humoral immune response to Pn in $TLR2^{-/-}$ mice suggested that TLR2 might play an important role directly at the level of adaptive immunity (i.e. DC, T cells and/ or B cells). More specifically we examined: 1) whether the lack of TLR2 on the DC affected its ability secrete cytokines, undergo phenotypic maturation in vitro, and induce a type 1 humoral response when pulsed with Pn14 in vitro and adoptively transferred into naïve mice and 2) determine whether B and /or T cell expression of TLR2 played a significant role in stimulating B

and T cells in vitro in response to Pn14, and whether TLR2 was critical for induction of a humoral response to Pn14 in vivo.

Hypothesis #2: Do Pn-pulsed MΦ actively elicit a humoral response following transfer into naïve mice?

Our lab has demonstrated that immature bone marrow derived dendritic cells (BMDCs) mature and secrete cytokines in response to Pn14 and upon adoptive transfer into naïve mice induce a primary Ig response specific for Pn proteins and polysaccharides. Pulsed BMDCs also induce immunologic memory which is most pronounced for the anti-protein response. In addition to a requirement for viable BMDCs, the protein-and polysaccharide-specific IgG responses induced by pulsed BMDCs also share a requirement for T cells and B-7 dependent costimulation, whereas the IgM anti-polysaccharide response is relatively less dependent on these forms of help (43).

Numerous studies have provided compelling evidence that dendritic cells (DC) are unique in their capacity to prime naïve T cells (221, 222). The role of other antigen-presenting cells (APC), such as macrophages and B cells, is thought instead to be primarily the promotion of various effector functions of T cells previously primed by DC. However most of the studies were exclusively focused on the ability of DC to prime naïve T cells and did not focus the ability of MΦ to induce a primary antibody response in vivo. In fact the only report to date investigating the role of MΦ to elicit a primary humoral response suggested that MΦ might be able to induce a humoral response in a

naïve animal (223). However it was unclear whether the MΦ was playing an active role as an APC or transferring the Ag to another APC (223).

Collectively these data led us to examine a potential active role for the MΦ in eliciting a humoral response in a naïve animal when challenged with Pn. Specifically, we examined: 1) whether in vitro Pn pulsed-MΦ are able to elicit a humoral immune response following transfer into naïve mice 2) whether the MΦ is playing an active part in this response, 3) whether the humoral response is dependent on CD4+ T cells and 4) if the humoral response requires surface expression of CD40 and MHC class II on the MΦ.

Chapter 2

Submitted as:

S. Vasilevsky, G. Chattopadhyay, T-J Yeh, Q. Chen, G. Sen, and C. M. Snapper. **B and CD4+ T cell expression of TLR2 is critical for optimal induction of a T cell-dependent humoral immune response to intact *Streptococcus pneumoniae***

Abstract

TLR2-/- mice immunized with intact Streptococcus (Pn) elicit normal IgM, but defective CD4+ T cell-dependent (TD) type 1 IgG isotype production. Of note, this is associated with a largely intact innate immune response. Thus we studied the TD phosphorylcholine (PC)-specific IgG3 versus the T cell-independent IgM response to Pn to determine whether TLR2 acts directly at the level of the adaptive immune system. We demonstrate that bone marrow dendritic cells (BMDC) from TLR2-/- mice have only a modest defect in cytokine secretion, but undergo normal maturation in response to Pn. TLR2-/- BMDC pulsed with Pn and transferred into naive WT mice elicit a normal IgM and IgG3 anti-PC response, relative to WT BMDC. Pn is synergistic with multivalent B cell receptor crosslinking for DNA synthesis in purified WT B cells, but completely fails to costimulate activated TLR2-/- B cells. Similarly, Pn is synergistic with anti-CD3+ anti-CD28 mAbs for proliferation of purified WT CD4+ T cells, that is partially dependent on TLR2, whereas Pn primes TLR2-/- mice for a normal CD4+ T cell IFN-γ recall response. TLR2-/- B cells transferred into RAG-2-/- mice with WT CD4+ T cells, or TLR2-/- CD4+ T cells transferred into athymic nude mice, each elicit a significantly reduced IgG3 anti-PC response to Pn, in contrast to equivalent induction of PC-specific IgM relative to WT cells. These data are the first to demonstrate a major role for B and CD4+ T cell expression of TLR2 for eliciting anti-bacterial humoral immune response.

Introduction

Initial host defense against infections by extracellular bacteria is mediated by innate immunity, which is characterized by the use of germline-encoded pattern recognition receptors (PRRs) that bind distinct pathogen-associated molecular patterns (PAMPs) on microorganisms. The major PRRs in mammalian species are the Toll-like receptor (TLR) family of proteins (224-226). Currently, 12 TLRs are described in the mouse. TLRs mediate the activation of nuclear transcription factors via one or more adaptor proteins, most critically MyD88. The ultimate result of TLR signaling is transcriptional activation of numerous genes, including those encoding various proinflammatory cytokines, chemokines, and immune receptors. A major consequence of innate immune activation is the stimulation of dendritic cells (DC) that have internalized microorganisms at the site of infection (227). Activated DC migrate to secondary lymphoid organs, undergo phenotypic maturation, present peptide/MHC complexes and secrete cytokines that promote T cell activation and differentiation (228). In this manner, the innate immune system plays a major role in initiating and guiding the adaptive immune system.

Streptococcus pneumoniae [Pn], a Gram-positive extracellular bacteria, is the most important etiological agent of community-acquired pneumoniae and a major cause of morbidity and mortality in humans (229, 230). Pn is known to contain ligands for TLR2, TLR4, TLR7/8, and TLR9 that can act collectively to initiate innate immunity. This is largely mediated through phagocytosis and intracellular killing by neutrophils and macrophages which are recruited to, and activated at, the site of infection (231). Pn also

elicits antibody specific for various structures on the bacterial surface, which includes the capsular polysaccharide PS [PPS] (232), the cell wall C-polysaccharide (C-PS, teichoic acid), including it's phosphorylcholine (PC) moieties. (233, 234) and various pneumococcal proteins (235) including pneumococcal surface protein (PspA) (236) . Adaptive immunity to pneumococcus is largely conferred by these antibodies, which synergize with the innate immune system to confer markedly enhanced host protection (22, 237-239).

Mice genetically deficient in TLR2 (TLR2-/-) exhibit an increased (240-242) or no apparent (243, 244), sensitivity to infection with Pn, depending on the model system, in contrast to the more severe defect in host defense observed in MyD88-/- mice (244-246). In particular, we previously demonstrated that spleen cells from TLR2-/- mice elicit a largely normal cytokine and chemokine response in vitro and in vivo in response to Pn, capsular type 14 (Pn14) associated with no increase in lethality upon i.p. infection, relative to wild-type (WT) mice (244). More recently we demonstrated that TLR2 synergizes with both TLR4 and TLR9 for induction of the MyD88-dependent splenic cytokine and chemokine response to Pn14 (63). In contrast to their largely intact innate response to Pn14, TLR2-/- mice exhibited a significant decrease in the elicitation of type 1 IgG isotypes (IgG3, IgG2b, and IgG2a) specific for PPS, PC and a number of Pn proteins, whereas induced serum titers of specific IgG1 were equivalent to that observed in WT mice. Thus, the defective type 1 humoral immune response to Pn in TLR2-/- mice suggested that TLR2 might be playing an important role directly at the level of the adaptive immune system.

We previously demonstrated that the IgG, but not IgM, anti-PC and anti-PPS14 response to immunization with heat-inactivated Pn14, was dependent on CD4+ T cells, similar to that observed for the IgG response specific for several pneumococcal proteins (50, 217, 218). Although TLRs have been shown to be critical in mediating innate immune cell activation, they are also expressed by cells participating directly in adaptive immunity (i.e. B cells (247), CD4+ T cells (248-251) and DC (252)) and could therefore possibly play a more direct role in an adaptive immune response. Indeed, two recent reports have indicated a critical role for B cell expression of MyD88 and TLR4 (253), or TLR9 (254) in promoting the humoral response to model protein antigens or virus-like particles, respectively. In this report we extend these studies by analyzing the T cell-independent IgM anti-PC and T cell-dependent IgG3 anti-PC response to intact Pn14. We demonstrate for the first time that both B cell and CD4+ T cell expression of TLR2 is critical for optimal induction of humoral immunity in response to an intact bacterium.

Materials and Methods

Mice. C57BL/6 and B6129SF2/J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). $TLR2^{-/-}$ mice (255), produced in the laboratory of Dr. S. Akira (Osaka U., Osaka, Japan) were kind gifts from Dr. S. Akira (B6.129 background) and D. Golenbock [U. Mass. Med Ctr, Worcester, MA] (C57BL/6 background, backcrossed 9x). For genotyping of $TLR2^{-/-}$ mice, DNA was prepared from mouse tail snips. The primers and conditions used for genotyping by PCR are as follows: 1) TLR2 (B6.129), A: 5'GTT TAG TGC CTG TAT CCA GTC AGT GCG 3'; B: 5' AAT GGG TCA AGT CAA CAC TTC TCT GGC 3'; C: 5' ATC GCC TTC TAT CGC CTT CTT GAC GAG 3' (94°C for 1 min; 35 cycles of: 94°C for 30 s, 67°C for 30 s, and 72°C for 1 min; and 72°C for 10 min. For detection of the mutated allele, we used primers B and C. For the wild-type allele, we used primers A and B. The amplified products are both ~1,200 bp); 2) TLR2 (C57BL/6), A: 5' CAT TGA CAA CAT CAT CGA T 3'; B: 5' GTA GGT CTT GGT GTT CAT T 3' (94°C for 3 min; 12 cycles of: 94°C for 20 s, 64°C for 30 s, and 72°C for 35 s, followed by 25 cycles of: 94°C for 20 s, 58°C for 30 s, and 72°C for 35 s; and 72°C for 2 min). Both female and male mice were bred and maintained at U.S.U.H.S. in a pathogen-free environment and were used between 7-12 weeks of age. The experiments in this study were conducted according to the principles set forth in the Guide for the Care and Use of Laboratory Animals, Institute of Animal Resources, National Research Council, Department of Health, Education, and Welfare (National Institutes of Health) 78-23.

Reagents. Dextran-conjugated anti-IgD (αδ-dex) was prepared by conjugation of a "b" allotype-specific anti-IgD mAb (clone AF3) to dextran ($2x10^6$ MW) (256). PC-keyhole limpet hemocyanin (KLH) was synthesized as described previously (257). The resulting conjugate had a substitution degree of 19 PC/KLH. Both reagents were kind gifts of Dr. A. Lees (Lees BioConsulting, Gaithersburg, MD). A protein extract from *S. pneumoniae*, capsular type 14 (Pn14) was prepared using B-PER (Bacterial Protein Extraction Reagent) [PIERCE, Rockford, IL]. The following mAbs were obtained from BD Pharmingen (San Diego, CA): APC-rat IgG2a, κ anti-mouse CD90.2 (clone 53-2.1), PE-rat IgG2a, κ anti-mouse CD4 (clone H129.19), FITC-rat IgG2a, κ anti-mouse CD45R/B220 (clone RA3-6B2), Syrian hamster IgG2, λ1 anti-mouse CD28 (clone 37.51), and Armenian hamster IgG1, κ anti-mouse CD3ε (clone 145-2C11)

Preparation and immunization of *S. pneumoniae*, capsular type 14 (Pn14). A frozen stock of Pn14 was thawed and sub-cultured on BBL pre-made blood agar plates (VWR International, Bridgeport, NJ). Isolated colonies on blood agar were grown in Todd Hewitt broth (Becton Dickinson, Sparks, MD) to mid-log phase, collected, and heat killed by incubation at 60°C for 1h. Sterility was confirmed by subculture on blood agar plates. After extensive washings, the bacterial suspension was adjusted with PBS to give an absorbance reading at 650 nm of 0.6 which corresponded to 10^9 CFU/ml. Bacteria were then aliquoted at 10^{10} colony-forming units (CFU)/ml and frozen at –80°C until their use as antigen for mouse immunizations. Mice were immunized i.p. with 2×10^8 CFU of heat-killed bacteria in 250 μL of PBS. The Pn14 stock was tested for endotoxin using the Limulus Amebocyte Lysate assay (QCL-1000) from BioWhittaker

(Walkersville, MD). This assay demonstrated that mice injected with $2x10^8$ CFU equivalents of heat-killed Pn14 receive <20 pg of endotoxin. Serum samples for measurement of anti-PC Ab titers were prepared from blood obtained through the tail vein.

Purification of splenic B cells and CD4+ T cells. Single cell suspensions from spleen were prepared, and RBCs were lysed using ACK lysing buffer (Quality Biological, Inc., Gaithersburg, MD). B cells were positively selected by magnetic bead sorting using anti-mouse CD45R (B220) micro magnetic beads (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. Cell purities were checked by flow cytometry following each purification using a BD-LSR-II flow cytometer (BD Biosciences, San Jose, CA) and found to be 90-92% B220+ cells. CD4+ T cells (CD4+CD90+B220-) were purified by electric cell sorting using a BD Biosciences FACSAria flow cytometer cell sorter. Purities of >99% CD4+ T cells were obtained.

Measurement of DNA synthesis by [^{3}H]-TdR incorporation-Purified B220+ splenic B cells were cultured (2.5×10^5 cells/ml in 0.2 ml) in medium (RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 0.05 mM 2-ME, 50 μg/ml penicillin, and 50 μg/ml streptomycin), in the presence of various stimuli, in flat-bottom 96 well Costar plates (Corning Incorporated, Corning, NY). After 48 h in culture at 37°C in a 5% $C0_2$-containing incubator, [^{3}H]-TdR (2 μCi; specific activity of 25Ci/mmol or 925GBq/mmol, Cat#TRK120) [Amersham Biosciences, Piscataway, NJ] was added to the cultures for an additional 18 h. Cultured cells were then harvested onto

glass filter paper (Wallac, Turku, Finland, Cat # 1450-421) using a Harvester 96 (Tomtec, Hamden, CT). Specific incorporation of [^{3}H]-TdR was determined using a 1450 Microbeta,"Wallac" Trilux scintillation counter.

Production and culture of bone marrow dendritic cells (BMDC). BMDC were prepared as previously described (258). Briefly, bone marrow from femur and tibia was flushed, homogenized and the red blood cells were removed by lysis with ACK lysing buffer. The BM cells obtained were cultured at 1.25 x 10^6 cells/ml (24-well plates) in cell culture medium (RPMI 1640 supplemented with 5% FBS, 10,000 IU penicillin & 10μg/ml streptomycin, 1 mM sodium pyruvate, 2 mM L-glutamine, 0.1 mM nonessential amino acids, and 25 mM HEPES) supplemented with 10 ng/ml murine rGM-CSF (Sigma, St. Louis, MO). After 6-8 days of culture, non-adherent cells were harvested and were found to be 90% CD11c+ cells by flow cytometry. For in vitro studies, BMDC were plated in medium without GM-CSF, at 1 x 10^6 cells/ml in 24-well cell culture plates (Costar, Corning, NY). After 30 min, to allow cells to settle, varying concentrations of heat-killed Pn14 were added to the cultures. After 24 h, SN was obtained for measuring concentrations of secreted cytokines, and BMDC were harvested for flow cytometric analysis of cell surface phenotype markers.

Adoptive transfer of Pn14-pulsed BMDC The method of Pn14-pulsed BMDC transfer into naïve mice has been described in detailed elsewhere (258). Briefly, BMDC at 1 x 10^6/ml were incubated for 5 h in vitro with 8 x 10^8 CFU equivalents of heat-killed Pn14. Free bacteria was then removed from the BMDC cultures by washing ~6X in cold PBS, each time following by centrifugation for 10 min at 350 x *g* at 4^{o}C. A control tube

containing a mixture of thymocytes and CM-DiI fluorescent-labeled Pn14 at the same initial bacterial density was used to monitor the progress of the washings. If >100 free bacteria were found in pelleted cells, using an inverted fluorescent microscope, the washings were continued. Pn14-pulsed BMDC were then resuspended in fresh medium at 1 x 10^6 BMDC/200μl, and 200 μl were injected i.v. into each mouse. Serum samples were obtained 7 and 14 days after BMDC transfer.

Adoptive transfer of B cells and CD4+ T cells in RAG-$2^{-/-}$ mice. Single spleen cell suspensions were obtained and treated with ACK lysing buffer to remove red blood cells. Purified B and CD4+ T cells were obtained by positive selection using anti-B220 and anti-CD4 MACS beads, respectively, according to manufacturer's instructions (Miltenyi Biotec, Auburn, CA). Cell purities of ~90% were typically observed, using flow cytometry. RAG-$2^{-/-}$ mice were injected i.p. with a mixture of 2 x 10^7 B cells (derived from either wild-type or TLR$2^{-/-}$ mice) and 1 x 10^7 CD4+ T cells (derived from wild-type mice). Mice were then immunized 18 h later with 2 x 10^8 CFU equivalents of heat-killed Pn14 and sera were obtained 7 and 14 days later.

CD4+ T cell priming assay (259). Mice were immunized i.p. with 2 x 10^8 CFU heat-killed Pn14 in saline and similarly boosted on day 14. On day 28, spleen cell suspensions were prepared from individual mice and RBCs were removed using ACK lysing buffer. Spleen cells (1x10^7) from either naïve or primed wild-type or TLR$2^{-/-}$ mice were suspended in 2 ml of culture medium in 24-well plates and cultured at 37°C in an atmosphere of 7% CO2 and 95% humidity. After 30 min incubation, 30 micrograms of Pn14-derived protein extract was added to each culture well. At 72 h SN was obtained for

measurement of secreted IFN-γ concentrations by ELISA. Cytokine secretion was eliminated when spleen cells were first depleted of $CD4^+$ T cells using anti-CD4-coated magnetic beads (259).

Measurement of cytokine concentrations in culture SN by ELISA. The concentrations of specific cytokines released into the medium of BMDC cultures were measured using optimized standard sandwich ELISA. Recombinant cytokines used as standards, as well as the capture mAbs, biotinylated mAbs used for detection, and streptavidin-alkaline phosphatase (AP), were purchased from BD PharMingen (San Diego, CA). Streptavidin-AP was used in combination with *p*-nitrophenyl phosphate disodium (Sigma) as substrate to detect the specific binding. Standards were included in every plate, and the samples were tested in duplicate

Measurement of type 1 interferon. Type 1 interferon concentrations in culture SN were measured as previously described (260). Briefly, 100 μl of BMDC culture SN was added to 96 well plates pre-seeded with 40,000 L-cells in 100 μl of medium to make an initial 1:2 dilution. SN was further serially diluted by 2-fold and cultures were incubated overnight at 37°C. SN was removed, plates were washed with 100μl PBS and challenged with 100 μl encephalomyocarditis (EMC) virus at 10^{-3} dilution (multiplicity of infection of 0.1). Cultures were incubated overnight at 37°C until the viral cytopathic effect (CPE) reached 100% in medium-only control. The process was terminated with 20% formaldehyde and the monolayer was stained with 0.5% crystal violet to detect attached, viable cells. Plates were air dried and read in a plate reader equipped with a 540 nm filter. The reciprocal dilution of the well which exhibited a ~50% CPE was

defined as the antiviral titer. All titers were compared to a reference type 1 IFN, and converted to international units/ml (IU/ml).

Flow cytometric analysis. All steps were performed on ice. FcγR receptors were initially blocked by addition of 10 μg/ml of rat IgG2b,κ anti-mouse FcγRI/II/III (clone 2.4G2). Cells were then stained 20 min later by incubation for an additional 30 min with PE-Armenian hamster IgG1,λ2 anti-mouse CD11c (clone HL3) plus either FITC-mouse IgG2a,κ anti-mouse MHC-II^b (clone AF6 120.1), FITC-rat IgG2a,κ anti-mouse CD40 (clone 3/23), PE-rat IgG2a,κ anti-mouse CD86 (clone GL1), or Armenian hamster IgG2a,κ anti-mouse (clone 16-10A1). All mAbs were purchased from BD Pharmingen. Irrelevant isotype- and species-matched mAbs were used as staining controls. Cells were analyzed on an EPICS XL-MCL (Beckman Coulter, Miami, FL). Dead cells and debris were eliminated from analysis by excluding cells positive for propidium iodide and gating on the appropriate forward and side scatter profile.

Measurement of serum IgM and IgG3 anti-PC titers. Immulon 4 ELISA plates (Dynex Technologies, Inc., Chantilly, VA) were coated (50μL/well) with PC-KLH (5 μg/ml) in PBS for 1h at 37^{o}C or overnight at 4^{o}C. Plates were washed 3X with PBS + 0.1% Tween 20 and were blocked with PBS + 1% BSA for 30 min at 37^{o}C or overnight at 4^{o}C. Threefold dilutions of serum samples, starting at a 1/100 serum dilution, in PBS + 0.05% Tween 20 were then added for 1h at 37^{o}C or overnight at 4^{o}C and plates were washed 3X with PBS + 0.1% Tween 20. Alkaline phosphatase-conjugated polyclonal goat anti-mouse IgM or IgG3 Abs (200 ng/ml final concentration) in PBS + 0.05% Tween

20 were then added, and plates were incubated at 37°C for 1 h. Plates were washed five times with PBS + 0.1% Tween 20. Substrate (*p*-nitrophenyl phosphate, disodium; Sigma, St. Louis, MO) at 1 mg/ml in TM buffer (1 M Tris + 0.3 mM $MgCl_2$, pH 9.8) was then added for color development. Color was read at an absorbance of 405 nm on a Multiskan Ascent ELISA reader (Labsystems, Finland).

Statistics. Data were expressed as geometric mean ± S.E.M. of the individual results. Student's t-test was used to determine statistical significance between groups. $P<0.05$ was considered statistically significant.

Results

TLR2-/- mice elicit a defective IgG3, but normal IgM, anti-PC response to intact Pn14. We previously demonstrated that TLR2-/- mice (on a mixed B6.129 background) elicit a normal IgM, but defective type I IgG (IgG3, IgG2b, IgG2a) anti-phosphorylcholine (PC) response to intact *S. pneumoniae*, capsular type 14 (Pn14), relative to B6.129 controls (244). The IgG, but not IgM anti-PC response is dependent on TCR-α/β+ CD4+ T cells, B7-dependent costimulation, and CD40-CD40-ligand interactions (257, 261, 262). The defective type 1 IgG anti-PC response in TLR2-/- mice was associated with a largely intact innate immune response (244), suggesting a role for TLR2 directly at the level of adaptive immunity. Since IgG3 is the predominant IgG anti-PC isotype we studied the PC-specific IgM and IgG3 response to Pn14 to determine the mechanism of TLR2 action. As illustrated in Fig. 3 we confirmed our previous observations, this time using TLR2-/- mice extensively backcrossed onto the C57BL/6 background, and using C57BL/6 mice as wild-type (WT) controls. Thus, WT and TLR2-/- mice made an equivalent IgM anti-PC response following i.p. immunization with heat-killed Pn14. In contrast, TLR2-/- mice exhibited a significant ($p<0.05$) reduction (up to 4-fold) in serum titers of PC-specific IgG3.

TLR2-/- bone marrow dendritic cells (BMDC) elicit normal IgM and IgG3 anti-PC response to Pn14. We previously demonstrated that BMDC pulsed with Pn14 in vitro secrete cytokines (e.g. IL-12, IL-6 and TNF-α) and undergo phenotypic maturation

(258). Further, upon adoptive transfer into naïve WT mice, in vitro Pn14-pulsed BMDC elicit both anti-polysaccharide (including anti-PC) and anti-protein responses that are dependent on viable BMDC immediately prior to transfer, as well as T cells and B7-dependent costimulation in the naïve recipient. The absolute requirement for viable BMDC immediately prior to injection and for BMDC that are IL-6+/+ indicated that the transferred BMDC itself was playing an active role in this system, and not simply transferring antigen in a passive manner. We thus utilized this approach to determine the relative ability of in vitro Pn14-pulsed BMDC lacking TLR2 to elicit an IgM and IgG3 anti-PC response upon adoptive transfer into naïve WT recipients. As illustrated in Fig. 4A, Pam3Cys, a TLR2 ligand (263, 264) failed to induce TLR2-/- BMDC to secrete IL-6, IL-12, and TNF-α, relative to TLR2-/- BMDC cultured in medium alone, whereas it induced all 3 cytokines in WT BMDC. In contrast, cytokine secretion by TLR2-/- BMDC in response to LPS (TLR4 ligand) (265) or CpG-ODN (TLR9 ligand) (266) was largely equivalent to WT BMDC. These data thus confirmed that BMDC obtained from mice genotyped as TLR2-/-, were indeed specifically deficient in TLR2 function. TLR2-/- BMDC cultured with varying doses of heat-killed Pn14 in vitro were found to have a significant, though partial, defect in IL-6, IL-12, and TNF-α secretion, more apparent at lower Pn14 doses (Fig. 4B). In contrast, there were no significant differences in type 1 IFN secretion between WT and TLR2-/- BMDC (Fig. 4C). Of interest, both IL-12 (267) and type 1 IFN (268) have been implicated in stimulating type 1 humoral immune responses. In contrast to IL-6, IL-12, and TNF-α secretion, phenotypic maturation of WT and TLR2-/- BMDC induced by Pn14 was found to be essentially equivalent (Fig. 4D). Thus, Pn14 induced, in WT and TLR2-/- BMDC, the surface expression of MHC-II,

CD80, CD86, and CD40, molecules known to play an inductive role in APC-T cell interactions, to a similar degree. WT and TLR2-/- BMDC cultured for 5 h with Pn14 were then adoptively transferred into naïve syngeneic WT mice, following extensive washing to remove free Pn14. Sera obtained 7 and 14 days after BMDC transfer demonstrated no significant differences between WT and TLR2-/- BMDC in their capacity to induce an IgM or IgG3 anti-PC response (Fig. 5). Collectively, these data suggest that the defective type 1 IgG response in TLR2-/- mice is not critically related to altered DC function in response to Pn14.

TLR2-/- B cells fail completely to respond to the co-mitogenic effects of Pn14, whereas TLR2-/- CD4+ T cells are partially TLR2-dependent. Pn14 is known to express ligands for TLR2, TLR4, TLR7/8, and TLR9. We recently demonstrated that TLR2 synergizes with both TLR4 and TLR9 for induction of the MyD88-dependent spenic cytokine and chemokine response to Pn14 (63). We also previously demonstrated that B cells activated with dextran-conjugated anti-IgD antibodies (αδ-dex), a polyclonal model for PS-specific multivalent membrane Ig crosslinking, synergizes with several distinct TLR ligands for proliferation (269). We thus wished to determine whether Pn14 itself was directly co-mitogenic for αδ-dex-activated B cells. Purified splenic B cells (B220+) from either WT or TLR2-/- mice were stimulated for 48 h with Pn14 and/or αδ-dex and then pulsed overnight with ^{3}H-TdR for measurement of DNA synthesis. As illustrated in Fig. 6A, Pn14 alone had only a modest effect on inducing DNA synthesis by WT B cells that was nevertheless not observed for TLR2-/- B cells. In contrast, both WT and TLR2-/- exhibited an equally strong proliferative response to αδ-dex. Importantly, whereas Pn14 synergized with αδ-dex for DNA synthesis by WT B cells, it had no effect

on αδ-dex-activated TLR2-/- B cells. Thus, B cell expression of TLR2 is critical for the co-mitogenic effect of Pn14 on B cells activated via multivalent membrane Ig crosslinking.

We also wished to determine whether Pn14 directly stimulated DNA synthesis in purified WT or TLR2-/- CD4+ T cells in the absence or presence of sub-mitogenic stimulation with anti-CD3 + anti-CD28 mAbs. As illustrated in Fig. 6B, Pn14 alone failed to stimulate DNA synthesis in either WT or TLR2-/- CD4+ T cells relative to cells cultured in medium alone. Both WT and TLR2-/- CD4+ T cells elicited comparable mitogenic responses following stimulation with anti-CD3 + anti-CD28 mAbs. The combination of anti-CD3 + anti-CD28 mAbs and Pn14 resulted in a synergistic increase in DNA synthesis in both WT and TLR2-/- CD4+ T cells, although the Pn14-mediated costimulation was significantly ($p < 0.05$) lower in TLR2-/- CD4+ T cells relative to WT. Additional studies using MyD88-/- CD4+ T cells gave similar results to that observed using cells from TLR2-/- mice (data not shown), indicating that Pn14 directly costimulates CD4+ T cell mitogenesis in both a MyD88-dependent and independent manner.

CD4+ T cell priming for IFN-γ secretion in response to Pn14 in vivo is similar in WT and TLR2-/- mice. We previously demonstrated that in vitro stimulation of spleen cells, obtained from mice pre-immunized 2x with Pn14, with a Pn14-derived protein extract (PnP) elicits a significantly enhanced IFN-γ secretory response relative to spleen cells obtained from naïve mice (259). This secreted IFN-γ is derived exclusively from CD4+ T cells. In contrast, there is no detectable IL-4, IL-5, or IL-13 secretion when

using spleen cells from Pn14-primed WT mice, although IL-4-/- mice do exhibit a selective decrease in the vivo IgG1 response to Pn14 (270). Since IFN-γ can stimulate switching to IgG2a (271) and IgG3 (272) and is a hallmark of a type 1 cytokine response (273), we wished to determine whether Pn14-primed TLR2-/- mice exhibited diminished CD4+ T cell priming for IFN-γ. As illustrated in Fig. 7, spleen cells from Pn14-primed WT and TLR2-/- mice exhibited no significant differences in CD4+ T cell priming for IFN-γ secretion. Neither Pn14-primed WT nor TLR2-/- mice exhibit any detectable priming for IL-4, IL-5, or IL-13 (data not shown).

B cell and/or CD4+ T cell expression of TLR2 has a significant role in stimulating an optimal TD IgG3, but not TI IgM, anti-PC response to Pn14. The critical role of TLR2 on αδ-dex-activated B cells, and the partial dependence of TLR2 on anti-CD3 + anti-CD28-CD4+ T cells for mediating the co-mitogenic effect of Pn14, suggested a possible role for B cell and/or CD4+ T cells expression of TLR2 in the in vivo anti-PC response to Pn14. To determine this, we purified splenic B cells from WT and TLR2-/- mice and co-injected them i.p. into RAG-2-/- mice with WT CD4+ T cells, followed 1 day later by i.p. immunization with Pn14. RAG-2-/- mice exhibit a critical defect in VDJ recombination, and thus lack B cells and T cells. Sera were obtained 7 and 14 days later for determination of IgM and IgG3 anti-PC titers. As illustrated in Fig. 8A, no significant difference was observed in the ability of WT and TLR2-/- B cells to elicit an IgM anti-PC response. In contrast, WT B cells elicited a significantly higher IgG3 anti-PC response, on day 7 and 14, relative to TLR2-/- B cells in the RAG2-/- recipients. This difference was similar to that observed in intact WT mice immunized with Pn14 (Fig. 3). Adoptive transfer of WT B cells into RAG2-/- mice in the absence o WT CD4+

T cells resulted in 10-fold lower serum titers of IgG3 anti-PC relative to RAG2-/- mice receiving both WT B and CD4+ T cells (data not shown), confirming the T cell-dependence of this response in this experimental system.

We next purified WT and TLR2-/- CD4+ T cells and injected them i.p. into separate groups of athymic nude mice which exhibit a profound T cell deficiency. The injection of either WT or TLR2-/- CD4+ T cells had no significant effect on the Pn14-mediated induction of the IgM anti-PC response observed in athymic nude mice to which no CD4+ T cells were transferred (Fig. 8B), confirming the T cell-independence of this response. In contrast, injection of WT CD4+ T cells resulted in a significant ($p<0.05$) 5-fold enhancement in the IgG3 anti-PC response in Pn14-immunized athymic nude mice, relative to Pn14-immunized mice that received no cell transfer. In contrast, injection of TLR2-/- CD4+ T cells did not significantly enhance the Pn14-induced IgG3 anti-PC response relative to that observed in mice to which no CD4+ T cells were injected (Fig. 8B). Collectively, these data indicate an important role for both B cell and CD4+ T cell expression of TLR2 in stimulating a humoral immune response to Pn14, in the setting of a largely intact innate immune system.

Discussion

In this report we investigated the mechanism underlying the defective in vivo type 1 IgG isotype response of TLR2-/- mice to intact, heat-killed Pn14. Although the initial innate immune response to a pathogen is known to play a major role in guiding CD4+ T cell subset differentiation (228), we were intrigued by our earlier finding that the in vitro and in vivo splenic cytokine and chemokine response of TLR2-/- mice, including IL-12 secretion, upon Pn14 challenge was largely equivalent to WT mice (244). In addition, TLR2-/- mice exhibited no apparent increased susceptibility to i.p. challenge with live Pn14. In sharp contrast, the splenic cytokine and chemokine response to Pn14 was largely abrogated in MyD88-/- mice, which were concomitantly highly susceptible to i.p. challenge with live Pn14 (244), suggesting that TLRs in addition to, or other than, TLR2 were playing an important role. Indeed, we recently demonstrated that TLR2 is synergistic with TLR4 and TLR9 in the Pn14-induced MyD88-dependent splenic cytokine and chemokine response (274). Although MyD88-/-, like TLR2-/-, mice exhibited a defective type 1 IgG response to Pn14, the IgG1 (type 2) response, which is under positive regulation by endogenous IL-4 (270), was significantly elevated. This observation stood in contrast to the normal IgG1 response observed in TLR2-/- mice (244). In this regard, we earlier demonstrated that CD4+ T cells from MyD88-/- mice, primed in vivo with Pn14, secreted the type 2 cytokines, IL-5 and IL-13 following in vitro re-challenge with PnP, with no change in IFN-γ secretion, whereas WT mice made no detectable type 2 cytokines (259). Further, we demonstrated that CD4+ T cells from

Pn14-primed IL-1R-/- mice elicited a markedly defective IFN-γ recall response and no detectable IL-4, IL-5, or IL-13 response (259). This was associated with a marked defect in the elicitation of both type 1 and type 2 isotypes. Thus, we hypothesized that defective IL-12 secretion in MyD88-/- mice, likely from macrophages and DC, in response to Pn14 shifted T cell differentiation toward a type 2 response with a concomitant decrease in type 1 IgG isotypes and an increase in IgG1 (228), although a role for MyD88 expression in B cells and/or T cells was also possible. However, as discussed above, this mechanism seemed unlikely to explain the selective defect in the type 1 IgG response in TLR2-/- mice and suggested instead a possible requirement for TLR2 expression by B and/or T cells, or perhaps selectively in DC.

In light of the relatively high and easily detectable IgM and IgG3 anti-PC response to Pn14, and the selective decrease in IgG3 anti-PC serum titers in Pn14-immunized TLR2-/- mice, we studied this response in more detail to determine the mechanism for its dependence on TLR2. Whereas the IgM anti-PC response is T cell-independent, an optimal IgG3 anti-PC response requires TCR-α/β+CD4+ T cell help, including CD40/CD40-ligand interactions (257). We show that Pn14-pulsed TLR2-/- BMDC elicit a normal IgM and IgG3 anti-PC response when adoptively transferred into WT mice. In contrast to MyD88-/- mice (259), this was associated with no alteration in type 1 or type 2 cytokine secretion by in vitro-re-challenged CD4+ T cells, obtained from in vivo Pn14-primed TLR2-/- mice. Further, TLR2-/-, unlike MyD88-/- (275), BMDC underwent normal phenotypic maturation in response to Pn14 and secreted normal amounts of type 1 IFN, and substantial though somewhat reduced amounts of IL-12. Both these cytokines have been previously implicated in stimulating type 1 IgG responses in

vivo (267, 268). In contrast, αδ-dex-activated TLR2-/-, in contrast to WT, purified B cells failed to synergize with Pn14 for DNA synthesis, whereas the co-mitogenic effect of Pn14 on purified CD4+ T cells stimulated with sub-mitogenic doses of anti-CD3 + anti-CD28 mAbs was partially TLR2-dependent. These data confirm previous reports of TLR-mediated costimulation of DNA synthesis by TCR-activated CD4+ T cells (276). Most importantly, TLR2-/- B cells transferred into RAG-2-/- mice with WT CD4+ T cells or TLR2-/- CD4+ T cells injected into athymic nude mice, elicited a defective IgG3, but normal IgM anti-PC response to Pn14, relative to WT cells. These data thus implicate B cell and CD4+ T cell expression of TLR2 as critical for mediating an optimal type 1 IgG response to an intact extracellular Gram-positive bacterium. Our previous demonstration that IFN-γ can promote switching to IgG3 through induction of germline γ3 transcripts (277) is of interest in light of the normal Pn14-mediated T cell priming for IFN-γ secretion observed in TLR2-/- mice. In light of this, we speculate that the defective help delivered by TLR2 CD4+ T cells for the IgG3 anti-PC response perhaps reflects deficient T cell-mediated stimulation of B cell proliferation. A requirement for minimal number of rounds of proliferation for induction of the Ig class switch, depending on the isotype, has bee demonstrated (278).

TLR signaling in B cells has previously been shown in vitro to largely favor type I IgG class switching. Thus, murine B cells exposed to CpG-ODN, a TLR9 ligand, covalently linked to a protein antigen, act as effective APC for specific T cells, and induce Th1 differentiation via secretion of IL-12 (279). Additionally, CpG-ODN directly induce murine B cells to express germline C_H transcripts specific for IgG3, IgG2b, and IgG2a, and switching to these isotypes, whereas it inhibits IL-4-mediated induction of

IgG1 and IgE (280). Likewise, CpG-ODN stimulates human B cells to express $C_H\gamma1$, $C_H\gamma2$, and $C_H\gamma3$ germline transcripts and switch recombination, associated with an increase in activation-induced cytidine deaminase (AID) (281), an enzyme critical for promoting switch recombination (282). Membrane Ig crosslinking further ellicited IgG production in CpG-ODN-activated human B cells. LPS, a TLR4 ligand, selectively induces class switching to IgG3 and IgG2b in murine B cells, although human papillomavirus-like particles, also signaling via TLR4, induce switching to IgG of all isotypes (283). Stimulation of murine CD40-activated B cells via TLR7, utilizing R837, also selectively induces IgG2a/c secretion, which is further upregulated by type 1 IFN (284). Finally, murine B cells can be directly costimulated by a number of distinct TLR2 ligands, such as lipoprotein (285), Neisserial porins (286, 287), and macrophage-activating lipopeptide-2 (MALP-2) (288) to proliferate and differentiate into Ig-secreting cells. Whether TLR2 selectively induces type 1 IgG isotype expression in B cells is unknown. In this regard, lipoteichoic acid, which is expressed within the cell membrane of Pn, is known to bind and signal mammalian cells via TLR2 (289). Pn also expresses lipoproteins such as pneumococcal surface adhesin A (PsaA) (290), which could potentially engage TLR2 as well. Collectively, these data form the basis for the potential regulation of type 1 IgG responses, via direct B cell activation, in the absence of concomitant alterations in Th1 differentiation, as illustrated in this study.

Our data is consistent with two recent reports utilizing model protein antigens (291) or virus-like particles (292), and an experimental approach generally similar to ours. Thus, B cell-deficient (μMT) mice were immunized with human serum albumin (HSA) conjugated to LPS to induce HSA-specific memory Th cells (291). Purified B

cells from WT, TLR4-/-, or MyD88-/- mice were then transferred into the primed μMT mice followed by immunization with HSA-LPS. The IgM and IgG1 anti-HSA response, in mice receiving TLR4-/- or MyD88-/- B cells was reduced, relative to mice receiving WT B cells demonstrating a B cell-intrinsic requirement for TLR signaling. Similarly, MyD88-/- B cells transferred into μMT mice elicited a defective TD IgM and IgG1 anti-flagellin response upon flagellin immunization, although a normal TI IgG3 anti-flagellin response, relative to WT B cells (291). These data are analogous to ours in which B cell TLR2 expression was critical for the TD IgG3, but not TI IgM anti-PC response to Pn14. In another recent study, WT and TLR9-/- B cells were transferred into μMT mice and immunized with virus-like particles (VLP) loaded with CpG-ODN (292). The IgG2a, although not IgG1, anti-VLP response in mice receiving TLR9-/- B cells was significantly reduced relative to mice in which WT B cells were transferred. Of note, CpG-ODN is a type 1 adjuvant (293), likely accounting for the selective change in IgG2a. Our data is the first to implicate TLR2 and to utilize an intact bacterial pathogen, to confirm and significantly extend the concept of a key role for B cell expression of TLR in humoral immune responses. These data, to our knowledge, are also the first to implicate a role for any TLR expressed by CD4+ T cells in mediating an in vivo Ig response.

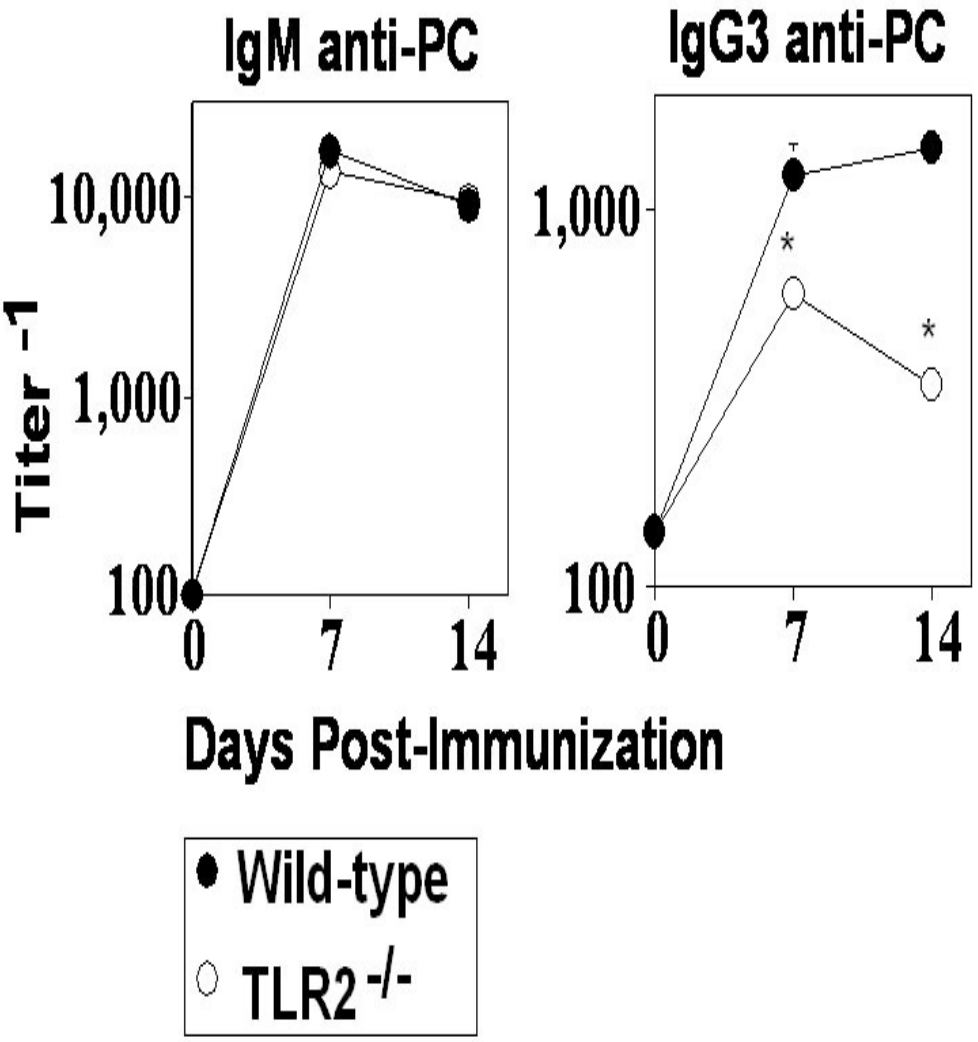

IgM anti-PC
IgG3 anti-PC
10,000
1,000
100
Titer -1
1,000
100
0
7
14
0
7
14
*
*
Days Post-Immunization
Wild-type
TLR2 -/-

Fig 3. TLR2-/- mice elicit a defective IgG3, but normal IgM, anti-PC response to intact Pn14. WT (C57BL/6) or TLR2-/- (C57BL/6 background) mice [seven per group] were injected i.p. with 2 x 10^8 CFU Pn14 in saline. Serum titers of PC-specific IgM and IgG3 were determined by ELISA on days 0, 7, and 14. Values represent the geometric mean ± SEM. *significant, $p<0.05$. The results are representative of two independent experiments.

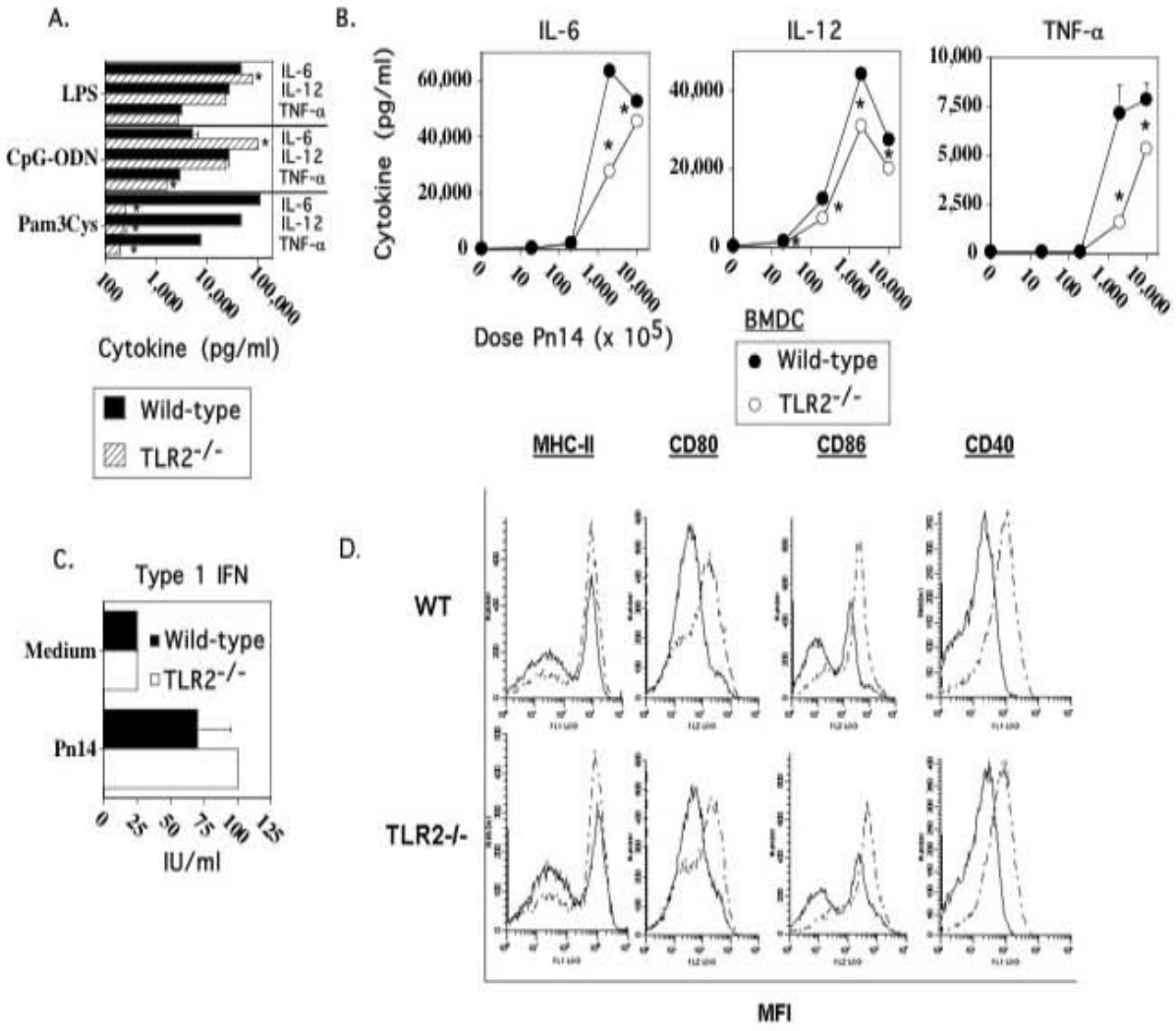

A.
LPS
CpG-ODN
Pam3Cys
IL-6
IL-12
TNF-α
100
1,000
10,000
100,000
Cytokine (pg/ml)
Wild-type
TLR2-/-
B.
IL-6
IL-12
TNF-α
Cytokine (pg/ml)
60,000
40,000
20,000
0
40,000
20,000
10,000
7,500
5,000
2,500
0
10
100
1,000
10,000
Dose Pn14 (x 10^5)
BMDC
Wild-type
TLR2-/-
MHC-II
CD80
CD86
CD40
C.
Type 1 IFN
Medium
Pn14
Wild-type
TLR2-/-
0
25
50
75
100
125
IU/ml
D.
WT
TLR2-/-
MFI

Fig 4. <u>Analysis of in vitro cytokine secretion and phenotypic maturation of Pn14-stimulated TLR2-/- and wild-type bone marrow dendritic cells (BMDC)</u>. BMDC (1 x 10^6/ml) from WT (B6.129) and TLR2-/- (B6.129 background) mice were incubated with **(A)** LPS (2μg/mL), CpG (2μg/mL), Pam_3CSK4 (150 ng/mL) or **(B)** varying doses of Pn14 for 24 h. Concentrations of secreted IL-6, IL-12 and TNF-α in culture SN were determined by ELISA. Values represent the geometric mean ± SEM. ∗significant, $p<0.05$. The results are representative of two independent experiments. **(C)** BMDC (1 x 10^6/ml) from WT (C57BL/6) and TLR2-/- (C57BL/6 background) mice were incubated with 2 x 10^8 CFU Pn14 for 24h. The concentration of type I IFN released into the culture SN was determined as an antiviral titer (IU/ml). Values represent the geometric mean ± SEM. The results are representative of two independent experiments. **(D)** BMDC from WT (B6129) and TLR2-/- (B6.129 background) mice were stimulated with 2 x 10^8 CFU/ml Pn14 for 24 h. The relative expression of MHC-II, CD80, CD86, and CD40 were determined by flow cytometry using specific mAbs. The results are representative of two independent experiments.

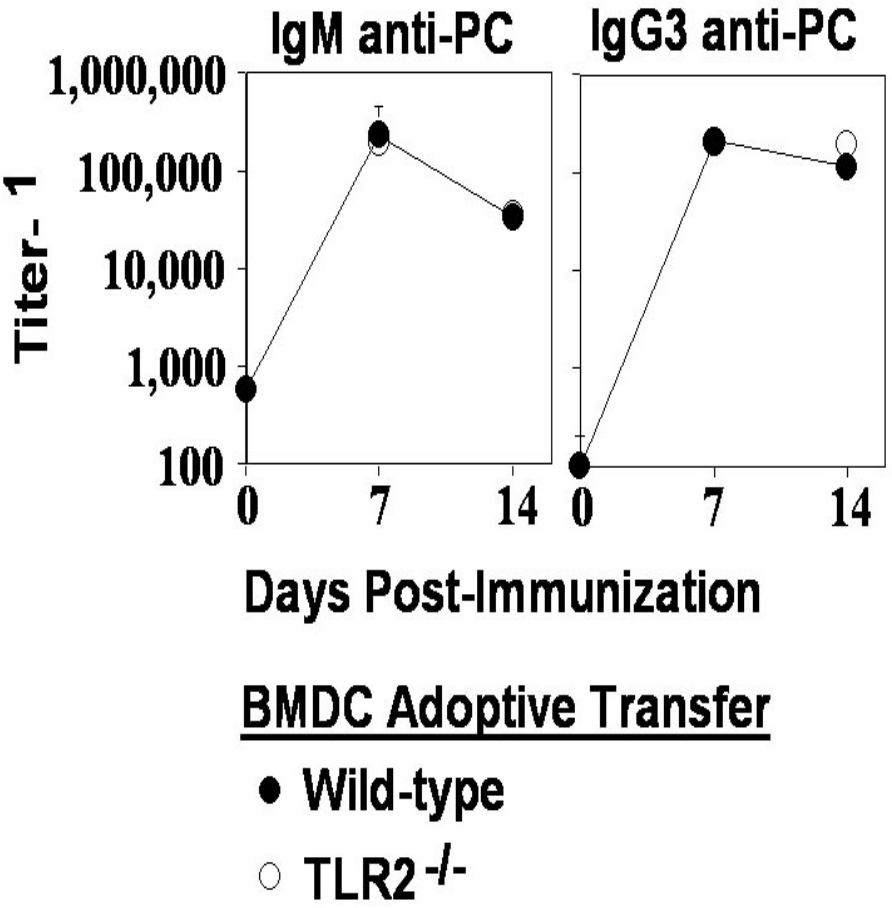

IgM anti-PC
IgG3 anti-PC
1,000,000
100,000
10,000
1,000
100
Titer- 1
0
7
14
0
7
14
Days Post-Immunization
BMDC Adoptive Transfer
Wild-type
TLR2 -/-

Fig 5. <u>TLR2-/- bone marrow dendritic cells (BMDC) elicit normal IgM and IgG3 anti-PC response to Pn14</u>. Pn14-pulsed WT (B6.129) and TLR2-/- (B6.129 background) BMDC were injected i.v. (1 x 10^6 BMDC per mouse) into WT (B6.129) mice (5 per group). Sera were collected at day 0, 7, and 14 for measurement of PC-specific IgM and IgG3 titers by ELISA. The data show the geometric mean ± SEM of the individual titers and are representative of two independent experiments. *significant, $p<0.05$.

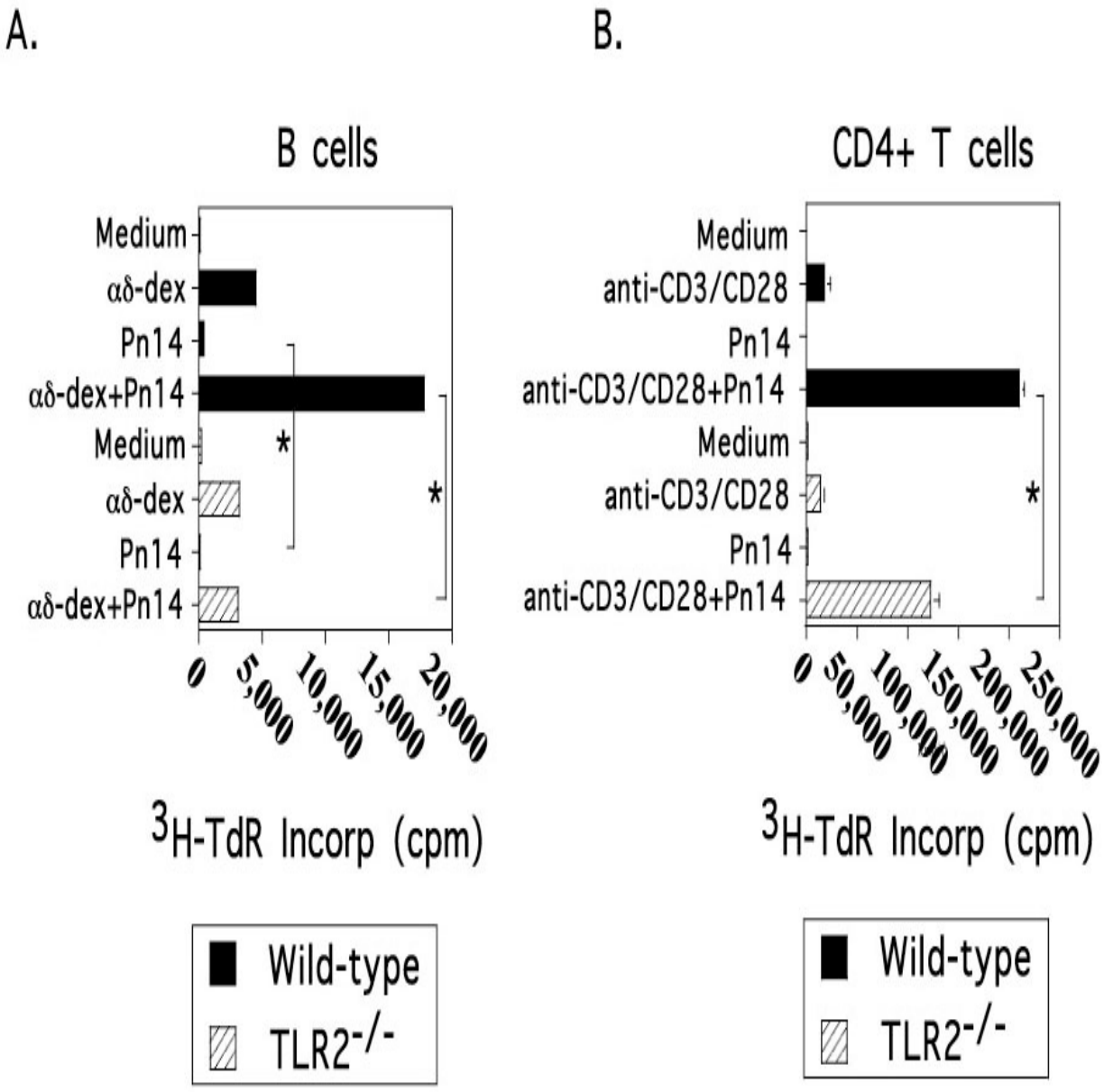

A.
B cells
Medium
αδ-dex
Pn14
αδ-dex+Pn14
Medium
αδ-dex
Pn14
αδ-dex+Pn14
0
5,000
10,000
15,000
20,000
*
*
3H-TdR Incorp (cpm)
Wild-type
TLR2-/-
B.
CD4+ T cells
Medium
anti-CD3/CD28
Pn14
anti-CD3/CD28+Pn14
Medium
anti-CD3/CD28
Pn14
anti-CD3/CD28+Pn14
0
50,000
100,000
150,000
200,000
250,000
*
3H-TdR Incorp (cpm)
Wild-type
TLR2-/-

Fig 6. <u>TLR2-/- B cells fail completely to respond to the co-mitogenic effects of Pn14, whereas TLR2-/- CD4+ T cells are partly TLR2-dependent</u>. (A). B cells from WT (B6.129) and TLR2-/- (B6.129 background) mice were cultured (2.5 x 10^5 cells/ml in triplicate wells) in absence or presence of Pn14 (2 x 10^8 CFU/ml) and/or αδ-dex (1 ng/ml) for 48 h. (B) CD4+ T cells from WT (C57BL/6) and TLR2-/- (C57BL/6 background) mice were cultured (1 x 10^6 cells/ml in triplicate wells) in the absence or presence of Pn14 (2 x 10^8 CFU/ml) and/or anti-CD3 mAb (0.3 μg/ml) + anti-CD28 (10 μg/ml) for 48 h. For both (A) and (B), ^{3}H-TdR was subsequently added for an additional 18h and cells were then harvested for 3H-TdR incorporation. The data show the geometric mean ± SEM of the individual wells. *significant, $p<0.05$. The results are representative of two independent experiments for both (A) and (B).

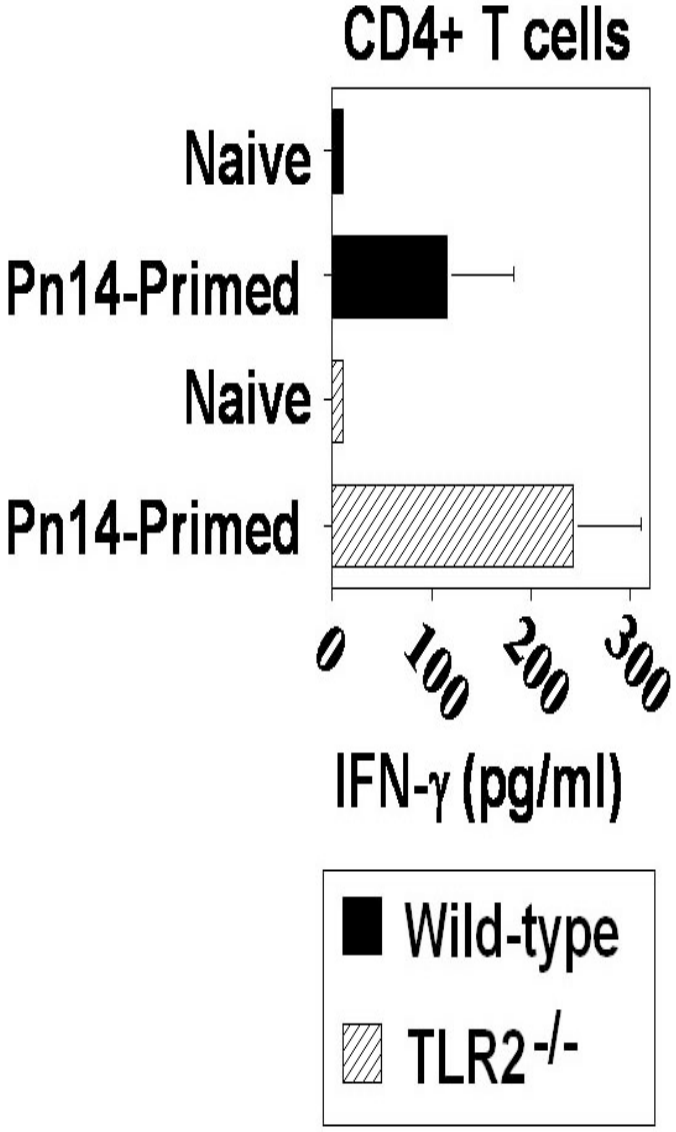

CD4+ T cells
Naive
Pn14-Primed
Naive
Pn14-Primed
0
100
200
300
IFN-γ (pg/ml)
Wild-type
TLR2 -/-

Fig 7. <u>CD4+ T cell priming for IFN-γ secretion in response to Pn14 in vivo is similar in WT and TLR2-/- mice</u>. WT (C57BL/6) and TLR2-/- (C57BL/6 background) mice were immunized i.p. with 2 x 10^8 CFU Pn14 and similarly boosted on day 14. On day 28 spleen cell suspensions were prepared from individual mice and cultured (triplicate wells) for 72 h with either medium alone or 30 μg/ml of Pn14-derived protein extract (PnP). Concentrations of IFN-γ, IL-4, IL-5, and IL-13 were measured by ELISA. The data show the geometric mean ± SEM of the individual SN for IFN-γ concentrations (IL-4, IL-5, and IL-13 were below detection in both groups [data not shown]). The results are representative of two independent experiments.

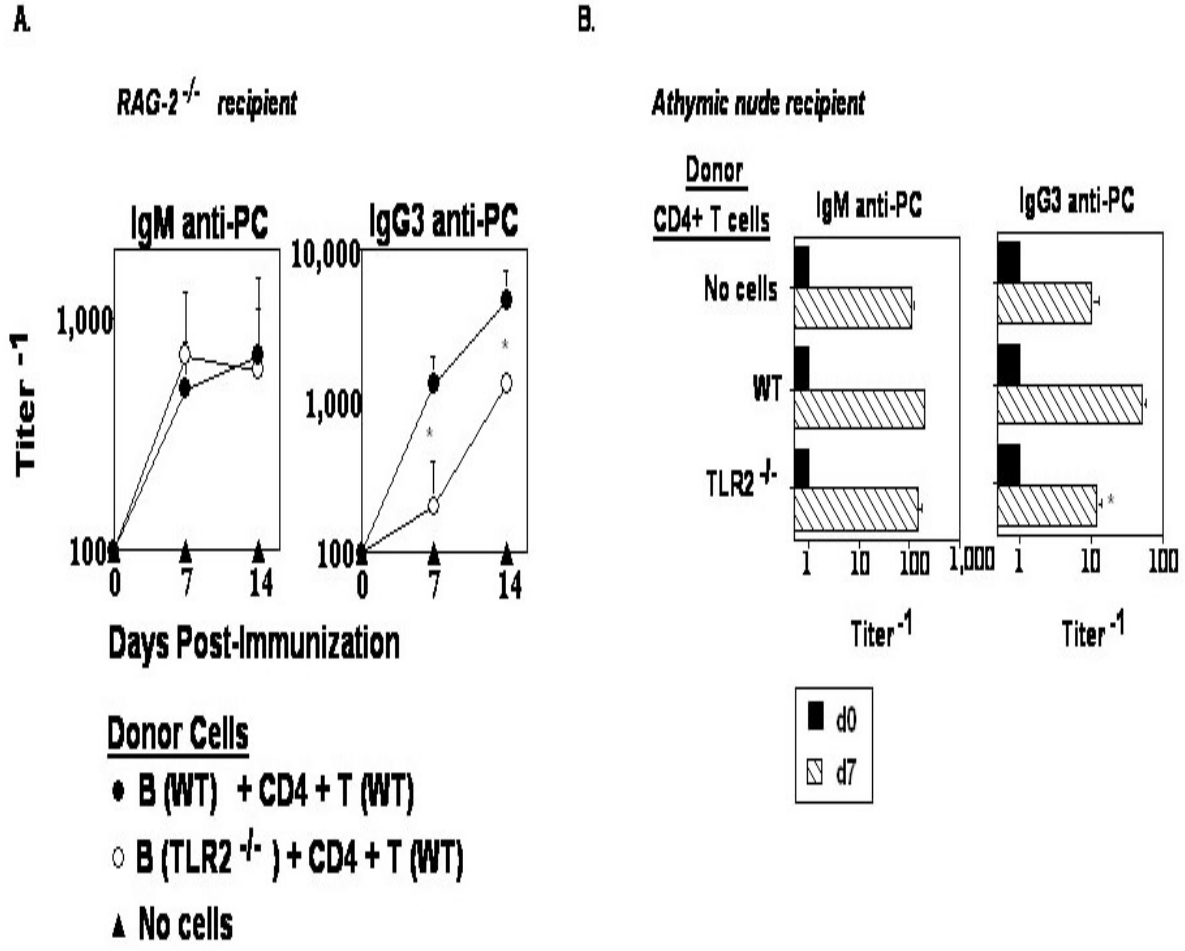

A.
RAG-2 -/- recipient
IgM anti-PC
IgG3 anti-PC
Titer -1
1,000
100
10,000
1,000
100
0
7
14
0
7
14
Days Post-Immunization
Donor Cells
B (WT) + CD4 + T (WT)
B (TLR2 -/-) + CD4 + T (WT)
No cells
B.
Athymic nude recipient
Donor
CD4+ T cells
IgM anti-PC
IgG3 anti-PC
No cells
WT
TLR2 -/-
1
10
100
1,000
1
10
100
Titer -1
Titer -1
d0
d7

Fig 8. <u>B cell expression of TLR2 has a significant role in stimulating the IgG3, but not IgM, anti-PC response to Pn14</u>. (A) RAG-$2^{-/-}$ (C57BL/6 background) mice (5 mice per group) were injected i.p. with 1.0 x 10^7 WT (C57BL/6) $CD4^+$ T cells + either 2.0 x 10^7 WT or TLR2-/- (C57BL/6 background) B cells. (B) Athymic nude (C57BL/6 background) mice (5 mice per group) were injected i.p. with 1.0 x 10^7 WT (C57BL/6) or TLR2-/- (C57BL/6 background) $CD4^+$ T cells. After 24 h, mice in both (A) and (B) were injected i.p. with 2 x 10^8 CFU Pn14 and sera was collected on days 0, 7, and/or 14. Serum titers of PC-specific IgM and IgG3 were determined by ELISA. Values represent the geometric mean ± SEM. *, $p<0.05$. The results are representative of two independent experiments for both (A) and (B).

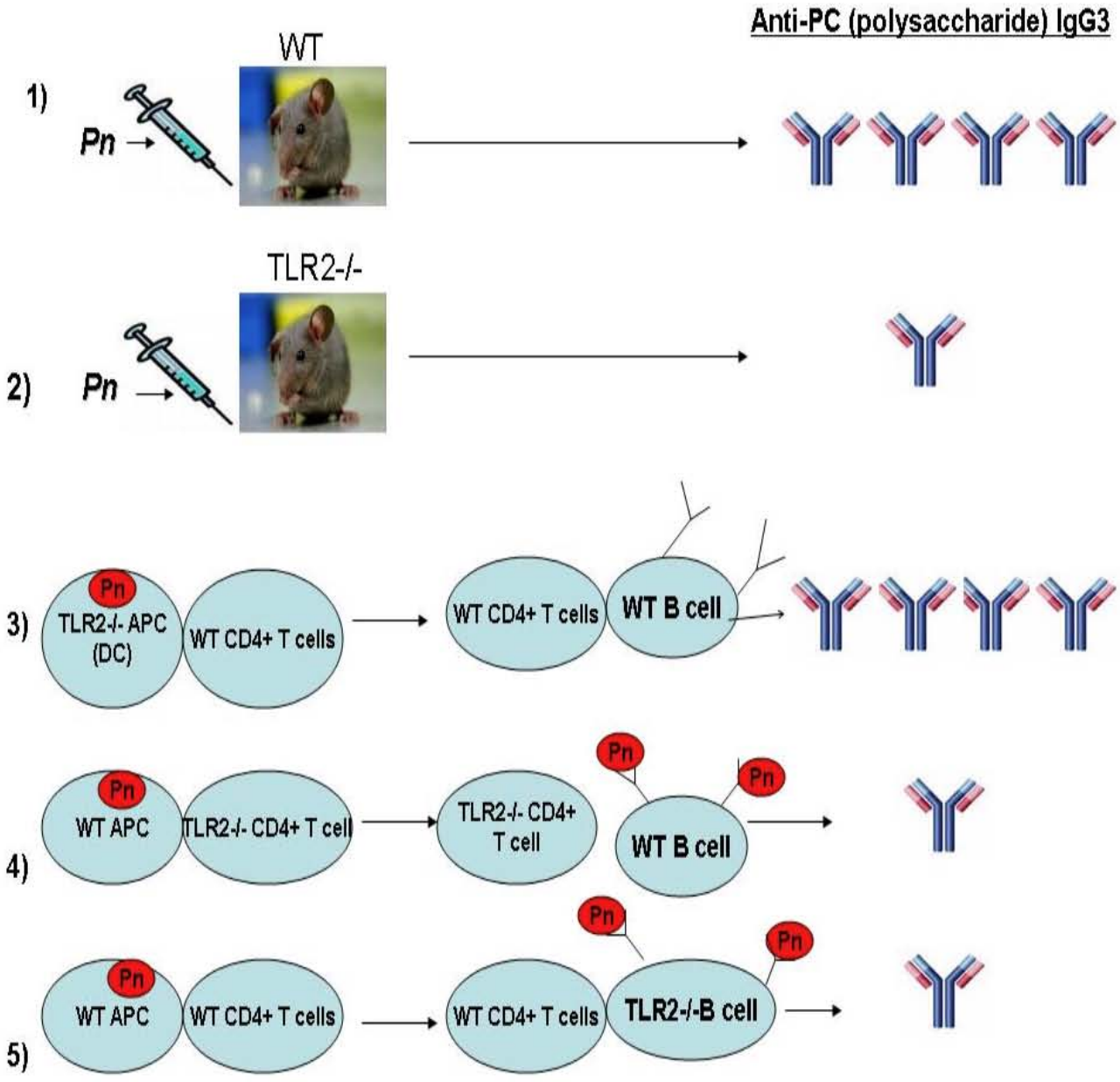

Anti-PC (polysaccharide) IgG3
WT
1)
Pn
TLR2-/-
2)
Pn
3)
Pn
TLR2-/- APC (DC)
WT CD4+ T cells
WT CD4+ T cells
WT B cell
4)
Pn
WT APC
TLR2-/- CD4+ T cell
TLR2-/- CD4+ T cell
Pn
Pn
WT B cell
5)
Pn
WT APC
WT CD4+ T cells
WT CD4+ T cells
Pn
Pn
TLR2-/-B cell

Fig 9. Summary: TLR2 expression on B and CD4+ T cells are critical for an optimal polysaccharide specific humoral response to *Streptococcus pneumoniae*

S. pneumoniae immunized TLR2-/- mice produce significantly lower amounts of polysaccharide specific (anti-PC) T_H1-associated (Type I) IgG3 compared to WT mice (1 & 2). WT mice injected with TLR2-deficient BMDC that were in vitro pulsed with *S. pneumoniae* were not defective in their ability to produce anti-PC IgG3 indicating TLR2 expression on APCs (i.e. DC) is not critical for an optimal anti-PC IgG3 response to *S. pneumoniae* (3). Adoptively transferred WT CD4+ T and TLR2-/- B cells into B and T deficient mice (RAG-/-) mice elicited a defective IgG3 anti-PC after immunization with *S. pneumoniae* (4). Athymic nude mice injected with TLR2-/- CD4+ T cells and challenged with *S. pneumoniae* were defective in their ability to elicit an IgG3 PC specific response (5). Conclusion: The expression of TLR2 on both B cells and CD4+ T cells is critical for a T_H1-associated (Type I) anti-polysaccharide humoral response to *Streptococcus pneumoniae*.

Chapter 3

Submited as:

Sam Vasilevsky, Jesus Colino, Roman Puliaev, David H. Canaday, and Clifford M. Snapper. **Macrophages pulsed with intact *Streptococcus pneumoniae* play an active role in eliciting a T cell-dependent, pneumococcal protein-specific antibody response upon adoptive transfer into naïve mice**

Abstract

It is well-established that macrophages are significantly less effective than dendritic cells (DC) at priming naïve CD4+ T cells, leading to the widespread belief that DC are unique in initiating T cell-dependent (TD) primary antibody responses in vivo. However, direct evidence for this is largely lacking. Thus, we compared the ability of DC and macrophages, pulsed in vitro with heat-killed *Streptococcus pneumoniae* (Pn), to elicit protein- and capsular polysaccharide (PPS)-specific Ig isotype production upon adoptive transfer into naïve mice. Bone marrow DC (BMDC) stimulated by Pn in vitro secreted significantly more pro- and anti-inflammatory cytokines, expressed higher levels of surface MHC-II and CD40, and presented intact Pn or recombinant pneumococcal surface protein A (PspA) to a PspA-specific T hybridoma more efficiently than either bone marrow macrophages (BMM) and/or peritoneal macrophages (PerM). Surprisingly, upon adoptive transfer into naïve mice, both Pn-pulsed BMM and PerM, elicited an IgM and/or IgG anti-PspA and anti-PPS response comparable in serum titers and IgG isotype distribution to that induced by BMDC. Whereas the IgG anti-PspA response elicited by Pn-pulsed BMM exhibited an absolute requirement for T cells in the recipient, the induced anti-PPS response was largely T cell-independent. Pn-pulsed BMM that were paraformaldehyde-fixed prior to transfer or lacking expression of MHC-II or CD40 were highly defective in eliciting an anti-PspA response when transferred into naïve, wild-type mice, although the anti-PPS response was largely unaffected. To our knowledge, these data are the first to provide compelling evidence that macrophages can play an active role in the induction of a T cell-dependent humoral immune response in a naïve host.

Introduction

Numerous studies have provided compelling evidence that dendritic cells (DC) are unique in their capacity to prime naïve T cells (221, 222). The role of other antigen-presenting cells (APC), such as macrophages and B cells, is thought instead to be primarily the promotion of various effector functions of T cells previously primed by DC. Thus, early studies demonstrated that DC were at least 100-fold more potent at stimulating a primary mixed leukocyte reaction (MLR) than macrophages or B cells (294-296). In addition splenic DC, but not peritoneal macrophages or unfractionated (B cell-rich) spleen cells, pulsed in vitro with a number of different soluble proteins, and injected into the footpads of mice, induced significant priming of CD4+ T cells from the draining lymph node, as evidenced by DNA synthesis in an in vitro restimulation assay (297). Similarly, in the presence of peptide antigen, splenic DC were significantly more efficient than splenic B cells, or peritoneal, splenic, or bone marrow-derived macrophages in promoting DNA synthesis or IL-2 production in naïve, peptide-specific transgenic CD4+ T cells in vitro (298, 299). However, it has also been demonstrated that macrophages are heterogeneous in their ability to present antigen to naïve CD4+ T cells (300). Thus, ~20% of murine splenic macrophage precursors were found to present native or peptide antigen to, and activate, naïve TCR transgenic CD4+ T cells. This correlated with the ability of presenting macrophages to release IL-12. DC have also been found to be the main APC for stimulating primed T cells in vitro, when cultured ex vivo from mice immunized with different soluble proteins; macrophages from immunized mice

were non-stimulatory and they were not able to transfer antigen to DC (301). Purified splenic DC, but not splenic or peritoneal macrophages, are also potent APC for restoring the in vitro T cell-dependent primary anti-SRBC response or TNP-KLH-induced anti-TNP response in mixtures of B and T cells (302, 303).

Consistent with the studies cited above, numerous studies have demonstrated the ability of antigen- or pathogen-pulsed DC to elicit immune responses upon adoptive transfer into naïve mice (304). However, little is known regarding a similar function for adoptively-transferred, antigen-pulsed macrophages. A series of studies by M. Moser and colleagues (223, 305-307) indeed demonstrated the ability of non-elicited peritoneal macrophages, as well as splenic DC, pulsed with soluble protein antigen and adoptively transferred into naïve mice, to both elicit specific antibody responses and T cell priming in vivo. In the absence of adjuvant during in vitro antigen pulsing of APC, macrophages elicited Th2, whereas DC induced Th1 responses upon adoptive transfer, although, in the presence of adjuvant the two responses were quantitatively and qualitatively similar. However, it is unclear from these studies whether the antigen-pulsed macrophage itself was playing an active and direct role in initiating immunity or perhaps was serving to transfer antigen to other, endogenous APC. This latter issue, however, was addressed by Pozzi et al who investigated the ability of adoptively-transferred peptide-pulsed bone marrow macrophages and DC to elicit CTL responses in transgenic CD8+ T cells specific for the peptide bound to MHC-I (308). Using the approach of adoptive transfer into bone marrow chimeras, both types of APC were shown to directly induce, without requiring peptide transfer to host APC, CD8+ T cell proliferation, cytokine secretion, and to promote differentiation into CTL and memory cells. Although macrophages injected s.c.

required pulsing with 10-fold more peptide than DC to elicit a comparable response, this was shown to be a function of lower migration of macrophages to lymph node, and not APC potency per se. Equivalent migration of APC to the spleen and subsequent CD8+ T cell responses were observed, however, using the i.v. route for APC injection.

Several in vivo studies support the notion that endogenous DC are indeed critical, non-redundant APCs for initiating immunity. Conventional (c)DC ($CD11c^{high}$) can be depleted by injection of diphtheria toxin into transgenic mice that express the DT receptor under the control of the CD11c promoter. DC depletion in these mice results in the abrogation of CTL priming in response to cross presentation of cell-associated (309) or exogenous soluble (310) antigen. Further CTL generation in response to Listeria monocytogenes and Plasmodium yoelii was also inhibited following DC depletion (309). Another study demonstrated that DC depletion in mice infected with lymphocytic choriomeningitis virus (LCMV) inhibited priming of LCMV-specific CD8+ T cells, despite the presence of LCMV in all major APC (DC, macrophages and B cells) (311). Priming of CD4+ T cells in response to i.v. injection of soluble or cell-associated protein is also inhibited by splenic cDC depletion, whereas lymph node plasmacytoid (p)DC can substitute for cDC for CD4+ T cell priming following s.c. immunization (310). Additionally, following DC-depletion, mice immunized i.v. with Mycobaterium tuberculosis (Mtb), exhibit a substantial delay in specific CD4+ T cell priming associated with impaired control of Mtb replication (312). Of interest, no studies have been reported to date on the effects of DC depletion on the induction of a T cell-dependent antibody response. Further, although both static and live imaging microscopy has clearly demonstrated the ability of antigen-expressing DC to form stable interactions with, and

activate, naïve CD4+ T cells (313), the ability of macrophages to mediate a similar function has not been resolved.

To our knowledge, it remains to be determined whether macrophages can play an active and direct role in initiating a CD4+ T cell-dependent humoral response in vivo. We previously demonstrated that mice immunized with intact *Streptococcus pneumoniae*, capsular type 14 (Pn14) elicit an IgG response specific for pneumococcal surface protein A (PspA) and type 14 capsular polysaccharide (PPS14) that is dependent on CD4+ T cells and CD40/CD40-ligand interactions (217). In contrast, the IgM anti-PPS14 response is T cell-independent. Further, we reported that BMDC pulsed with Pn14 in vitro and transferred i.v. into naïve, wild-type mice elicit IgG anti-PPS14 and IgG anti-PPS14 responses that are dependent on viable BMDC prior to transfer, their ability to secrete IL-6, and the presence of endogenous CD4+ T cells in the recipient mice (43). Whereas the IgG anti-PspA response required BMDC expression of MHC-II, CD40, and B7-1/B7-2, the IgG anti-PPS14 did not. Using this approach, we now wished to directly compare Pn14-pulsed BMDC with two distinct populations of macrophage (bone marrow macrophages [BMM] and peritoneal macrophages [PerM]) for their ability to induce anti-Pn humoral immunity upon adoptive transfer, and determine the mechanism by which this might occur. We show for the first time that macrophages pulsed with Pn14 can play an active, direct role in eliciting a specific humoral response in a naïve mouse, comparable to that observed using BMDC.

Materials and Methods

Mice. C57BL/6, BALB/c and athymic nude mice (BALB/c background) were obtained from the National Cancer Institute (Frederick, MD). $CD40^{-/-}$ mice (BALB/c background; strain name: $CNCr.129P2\text{-}Cd40^{tm1Kik}/J$) and MHC class $I1^{-/-}$ mice (C57BL/6 background, strain name: $B6.129\text{-}H2^{dlab1\text{-}E\alpha}/J$) were obtained from the Jackson Laboratory (Bar Harbor, ME). Both $CD40^{-/-}$ and MHC class $II^{-/-}$ male and female mice were bred and maintained at U.S.U.H.S. in a pathogen-free environment, and were used between 7-12 weeks of age. The experiments in this study were conducted according to the principles set forth in the Guide for the Care and Use of Laboratory Animals, Institute of Animal Resources, National Research Council, Department of Health, Education, and Welfare (National Institutes of Health) 78-23.

Reagents. Recombinant pneumococcal surface protein A (PspA) [family 1, seroclade 2] was expressed in *Sacharomyces cerevisiae* BJ3505 and purified as previously described (259). Purified pneumococcal capsular polysaccharide type 14 (PPS14) was purchased from the American Type Culture Collection (Manassas, VA.). Lipopeptide $Pam_3Cys\text{-}Ser\text{-}Lys_4$ (Pam_3Cys) and purified lipopolysaccharide from *E. coli* K12 strain (LPS) were purchased from InvivoGen (San Diego, CA). Phosphorotriated 30-mer (CpG-ODN) (AAA AAA AAA AAA AAC GTT AAA AAA AAA AAA) was synthesized in the Biomedical Instrumentation Center (U.S.U.H.S).

Preparation and immunization of *S. pneumoniae*, capsular type 14 (Pn14).

A frozen stock of *S. pneumoniae*, capsular type 14 was thawed and sub-cultured on BBL pre-made blood agar plates (VWR International, Bridgeport, NJ). Isolated colonies in blood agar were grown in Todd Hewitt broth (Becton Dickinson, Sparks, MD) to mid-log phase, collected, and heat killed by incubation at 60^{o}C for 1h. Sterility was confirmed by subculture on blood agar plates. After extensive washings, the bacterial suspension was adjusted with PBS to give an absorbance reading at 650 nm of 0.6 which corresponded to 10^9 CFU/ml. Bacteria were then aliquoted at 10^{10} CFU/ml and frozen at –80^{o}C until their use for mouse immunizations.

Creation of pneumococcal strain R6-14 (Pn-R6-14).

***Bacterial strains and growth culture media*:** The *S. pneumoniae* strain R6J (R6*cps*::Janus), a variant of the strain R6 in which the capsule locus is substituted by a Janus cassette, was kindly provided by Drs Kryzysztof Trzcinski and Marc Lipsitch (Harvard School of Public Health, Boston MA). All strains were cultured in Todd-Hewitt broth media (Difco) supplemented or not with 0.5% yeast carbon base (Difco, Beckton Dickinson, Sparks, MD) or in Trypticase soy agar supplemented with 5% Sheep blood (Beckton Dickinson). The selective media used was blood agar base supplemented with 5% defibrinated sheep blood (Hardy diagnostics, Santa Maria, CA) and antibiotics.

***Antibodies*:** An IgG1κ mouse monoclonal antibody specific for the capsular polysaccharide of *S. pneumoniae* type 14 (44.1) was kindly provided by Dr Alex Lucas (Children's Hospital Oakland Research Institute, Oakland, CA). Two IgG2a mouse monoclonal antibodies specific for the family 1, seroclade 2 of PspA (DC10-IA5 and

CF9IIB7), were kindly provided by Dr. Rick Schuman (Biosynexus, Inc., Gaithersburg, MD).

***Purification of bacterial genomic DNA*:** Chromosomal DNA was isolated by precipitation with isopropanol from the supernatant of lysed bacterial espheroplasts cleared by ammonium acetate precipitation (314)

***Construction of cps transformants*:** An isogenic variant of *S. pneumoniae* strain R6 expressing capsular polysaccharide type 14 (R6-14), was constructed by transformation with chromosomal DNA, essentially as described (315). Briefly, the Janus cassette of the strain R6J, used as a recipient, was replaced by the *cps14* locus by transformation with purified genomic DNA from Pn14, used as a donor. The Janus cassette comprises a kanamycin resistance marker and a counterselectable *rpsL*$^{+}$ marker that confers dominant streptomycin sensitivity in a streptomycin-resistant background (316). Transformation was induced by synthetic competence-stimulating peptide variant 1 (CSP-1) (317) synthesized by Dr. Micheal Flora (USUHS, Bethesda, MD). Transformants were selected by overnight growth on blood agar plates supplemented with 200 mg/liter of streptomycin. The expression of serologically intact Cps14 on the selected smooth colonies was confirmed by colony-blot using the mouse monoclonal antibody 44.1 specific for Cps14 as detection antibody. DNA from one isolate was purified and used to retransform R6J into a Cps14-expressing strain again. This process of backcross transformation was repeated three times in order to minimize uncontrolled recombinational replacements in loci other than *cps*. The final triple-backcross transformant (R6-14) exhibit a slightly faster rate of growth than the recipient R6J strain, but similar to the donor Pn14 strain, probably directly associated to the capsule

expression as previously noted (315). R6-14 expresses quantitatively equivalent levels of Cps14 relative to the donor strain (Pn14) as determined by quantitative sandwich ELISA. The PspA is serologically identical to the PspA of the recipient R6 strain.

Quantitation of PspA content of Pn strains R6 and R6-14: An inhibition ELISA was used to quantify bacterial PspA content. Briefly, serial dilutions of a suspension of intact, or lysed, bacteria were incubated overnight at 4^{o}C in PBS-1% BSA containing 1 ng/ml of the PspA-specific monoclonal antibody, DC10-IA5. In every assay, similar mixtures lacking the bacterial antigen were included as positive controls, and mixtures lacking the anti-PspA monoclonal antibody included as negative controls. A standard curve was generated for each assay using mixtures of serial dilutions of a preparation of PC-binding proteins, of known PspA content, purified from Pn strain R36A, and supplemented with 1 ng/ml of DC10-IA5.

After incubation, these suspensions were transferred to ELISA microtiter plates (Immulon 4HBX, Thermo Electron Corporation, Milford, MA) previously coated with 4 ng/well of recombinant PspA and blocked with PBS-2% BSA. The ELISA plates were then incubated overnight at 4^{o}C and washed five times with PBS-T. The amount of DC10-IA5 bound to the PspA-coated wells was detected by incubation with a conjugate of alkaline phosphatase and polyclonal goat IgG anti-mouse IgG (γ-chain) in combination with PNPP (p-nitrophenyl phosphate disodium; Sigma-Aldrich) as enzyme-substrate. The limit of detection of this ELISA was 3 ng/ml of PspA.

Establishment of PspA-specific T hybridomas. CD4+ T cell hybridomas specific for PspA peptide in association with MHC-II^d were produced and screened according to a previously published protocol (318). Briefly, BALB/c mice (Jackson Labs) were injected in the footpad with 50 μg of recombinant PspA emulsified in complete Freund's adjuvant (Sigma, St. Louis, MO). On day 7, a cell suspension was made from popliteal lymph nodes and cultured at 5 x 10^6 cells/well with soluble PspA at 5 μg/ml in 24-well plates. IL-2 (10 U/ml, Novartis, East Hanover, NJ) was added on days 2 and 3. Cells were then harvested on day 6 and fused, using polyethylene glycol (Boehringer Mannheim, Mannheim, Germany), with the fusion partner BW1100. Clones that grew in hypoxanthine/aminopterin/thymidine (HAT) (Sigma) selection media were screened for PspA-specificity and MHC restriction, by co-culture with bone marrow macrophages (BMM) or dendritic cells (BMDC) derived from BALB/c and C57BL/6 mice, in the presence or absence of PspA or cOVA. One T hybridoma, BALD4, which was CD4+CD8- by flow cytometry, and specific for, and highly sensitive to, PspA in association with MHC-II^d (data not shown) was selected for use in these studies. BALD4 was cultured in DMEM supplemented with 10% FCS (Hyclone, Logan, UT), 1 mM sodium pyruvate, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 25 mM Hepes, 50 μM 2-mercaptoethanol, 100 U/ml penicillin and 100 μg/ml of streptomycin. Except for the FCS, all the supplements were purchased from Gibco (Invitrogen, Carlsbad, CA). Mice were used in accordance with institutional guidelines, and approved by the Institutional Animal Care and Use Committee at Case Western Reserve University.

Preparation of BMDC. BMDC were prepared as previously described (43). Briefly, bone marrow from femur and tibia was flushed, homogenized and the red blood cells were removed by lysis with ACK lysing buffer (Gibco, Grand Island, NY). The BM cells obtained were cultured at 1.25 x 10^6 cells/ml (24-well plates) in RPMI 1640 + 5% FBS, 10,000 IU penicillin & 10μg/ml streptomycin, 1 mM sodium pyruvate, 2 mM L-glutamine, 0.1 mM nonessential amino acids, and 25 mM HEPES ("culture medium"), supplemented with 10 ng/ml of murine rGM-CSF (Sigma). After 7 days of culture, non-adherent cells were harvested.

Preparation of BMM. BMM were obtained using a similar approach to that for BMDCs, with slight modifications. After lysis with ACK buffer, bone marrow cells were cultured at 1 x 10^6 cells/ml in cell culture medium supplemented with 10 ng/ml of murine M-CSF (Sigma). Cells were plated in 6 well plates in a volume of 4-5 ml/well. On days 3 and 5, 3/4 culture media were removed and fresh culture media were added. On day 7, BMM were harvested by washing plates with sterile PBS to remove nonadherent cells. Cells were detached from the plate by adding 2 ml of detachment buffer (4 mg/ml lidocaine, 5 mM EDTA, PBS) for 3-5 minutes with pipetting.

Isolation of peritoneal macrophages (PerM). Culture medium (10 ml) was injected into the peritoneal cavity through the fat pad using a 23-gauge needle. After 3 minutes, lavage fluid containing the peritoneal cells was withdrawn using an 18-gauge needle. Cells were pelleted by centrifugation at 400 x g for 10 min. Red blood cells were removed by lysis with ACK lysing buffer. Cells were resuspended in culture medium, and cultured in 6 well plates (5 ml/plate) at 1 x 10^6 cells/ml overnight, followed by

washing of plates with sterile PBS to remove nonadherent cells. Adherent cells were then detached by adding 2 ml of detachment buffer (4 mg/ml lidocaine, 5 mM EDTA, PBS) for 3-5 minutes with pipetting.

In vitro culture of BMDC, BMM, and PerM. Cells were plated in culture medium without added GM-CSF or M-CSF, at 1 x 10^6 cells/ml in 24-well cell culture plates (Costar, Corning, NY). After 30 min, to allow cells to settle, varying concentrations of heat-killed Pn14 or Toll-like receptor (TLR) ligands were added to the cultures. After 24 h, SN was obtained for measuring concentrations of secreted cytokines, and cells were harvested for flow cytometric analysis of cell surface phenotypic markers.

APC stimulation of IL-2 production by PspA-specific T-cell hybridoma. BMDC and BMM derived from the same pool of BALB/c bone marrow cells, were collected after 7 days of culture, and resuspended in DMEM supplemented with 10% FCS. Both BMM and BMDC were plated at 2x10^4 APC/well in 96-well flat bottom tissue-culture plates (Costar, Lowell, MA), containing serial dilutions of PspA or heat-killed bacteria. After 2h of pulse with the antigen at 37^{o}C, the T cell hybridoma was added to the cultures at 10^5 cells/well and co-cultured with the APCs for 24h in the presence of free antigen. APCs derived from C57BL/6 bone marrow cells were used in every experiment as a control of MHC-restricted antigen presentation. Negative controls included cultures of the T-cell hybridoma and antigen in the absence of APCs, and APCs cultured with antigen in the absence of T-cell hybridoma.

Adoptive transfer of Pn14-pulsed APCs. BMDC, BMM, or PerM were cultured at 1 x 10^6 cells/ml for 4 h in the presence of 2 x 10^9 CFU of Pn14 in vitro. Free bacteria

were then removed from the cell cultures by washing ~6X in cold PBS, each time followed by centrifugation for 10 min at 350 x *g* at 4°C. A control tube containing a mixture of thymocytes and CM-DiI fluorescent-labeled Pn14 at the same initial bacterial density was used to monitor the progress of the washings. If >100 free bacteria were found in pelleted cells, using an inverted fluorescent microscope, the washings were continued. Pn14-pulsed cells were then resuspended in fresh medium at 1 x 10^6 cells/200 μl, and 200 μl were injected i.v. into each mouse. Serum samples were obtained 7, 14, and 21 days after cell transfer. On day 14, mice were boosted i.p. with 2 x 10^8 CFU of free heat-killed Pn14.

Measurement of cytokine concentrations in culture SN by ELISA. The concentrations of IL-6, IL-12, TNF-α, and IL-10 released into the culture medium by activated BMDC, BMM, and PerM, or IL-2 released by the APC/PspA-stimulated T hybridoma, were measured using optimized standard sandwich ELISA. Recombinant cytokines, used as standards, as well as the capture mAbs, biotinylated mAbs used for detection, and streptavidin-alkaline phosphatase (AP), were purchased from BD Pharmingen (San Diego, CA). Streptavidin-AP was used in combination with *p*-nitrophenyl phosphate disodium (Sigma) as substrate to detect the specific binding. Standards were included in every plate, and the samples were tested in duplicate

Flow cytometric analysis. All steps were performed on ice. FcγR receptors were initially blocked by addition of 10 μg/ml of rat IgG2b,κ anti-mouse FcγRI/II/III (clone 2.4G2). Cells were then stained 20 min later by incubation for an additional 30 min with either PE-Armenian hamster IgG1,λ2 anti-mouse CD11c (clone HL3), FITC-mouse

IgG2b,κ anti-mouse MHC-II^d (clone AMS-32.1), FITC-rat IgG2a,κ anti-mouse CD40 (clone 3/23), PE-rat IgG2a,κ anti-mouse CD86 (clone GL1), Armenian hamster IgG2a,κ anti-mouse CD80 (clone 16-10A1), or PE-rat IgG1,κ anti-mouse CD14 (clone rmc5-3). All mAbs were purchased from BD Pharmingen. FITC rat anti-mouse F4/80 (clone C1:A3-1) was purchased from Accurate Chemicals (Westbury, NY). Irrelevant isotype- and species-matched mAbs were used as staining controls. Cells were analyzed on an EPICS XL-MCL flow cytometer (Beckman Coulter, Miami, FL). Dead cells and debris were eliminated from analysis by excluding cells positive for propidium iodide and gating on the appropriate forward and side scatter profile.

Measurement of PspA and PPS14-specific serum Ig isotype titers. Immulon 4 ELISA plates (Dynex Technologies, Inc., Chantilly, VA) were coated (50 μL/well) with PSS14 (5 μg/ml) or PspA (2 μg/ml) in PBS for 1h at 37^{o}C or overnight at 4^{o}C. Plates were washed 3x with PBS + 0.1% Tween 20 and were blocked with PBS + 1% BSA for 30 min at 37^{o}C or overnight at 4^{o}C. Threefold dilutions of serum samples, starting at a 1/100 serum dilution, in PBS + 0.05% Tween 20 were then added for 1h at 37^{o}C or overnight at 4^{o}C and plates were washed 3X with PBS + 0.1% Tween 20. Alkaline phosphatase-conjugated polyclonal goat anti-mouse IgM, IgG, IgG1, IgG2a, IgG2b, or IgG3 Abs (200 ng/ml final concentration) in PBS + 0.05% Tween 20 were then added, and plates were incubated at 37^{o}C for 1 h. Plates were washed five times with PBS + 0.1% Tween 20. Substrate (*p*-nitrophenyl phosphate, disodium; Sigma, St. Louis, MO) at 1 mg/ml in TM buffer (1 M Tris + 0.3 mM $MgCl_2$, pH 9.8) was then added for color

development. Color was read at an absorbance of 405 nm on a Multiskan Ascent ELISA reader (Labsystems, Finland).

Fluorescent labeling of Pn14 with CM-DiI. Bacteria were labeled with the fluorescent lipophilic cell tracer chloromethylbenzamido derivative DiI (CM-DiI; Molecular Probes, Eugene, OR). Specifically, suspensions of 1 x 10^9 CFU of heat-killed Pn14 per milliliter were incubated at 37^0C for 10 min, in a solution containing 5 mM CM-DiI in PBS. The suspension was then incubated for an additional 30 min at 4^0C. After the incubation, extensive washings in culture media removed the excess of fluorescent tracer.

Phagocytic uptake of Pn14 by APCs. BMDC, BMM, or PerM were pulsed with varying ratios of CM-DiI-labeled Pn14. At different times (0, 10 min, 30 min, 1h, 3h, and 5h) the APCs were washed in PBS, and viable cells were analyzed by flow cytometry with gating according to size, to exclude free bacteria. APC pulsed with unlabeled bacteria at the same ratios were used as controls to determine the background of non-specific fluorescence. To block internalization of bacteria, and to control for cell surface binding of Pn14, cytochalasin D (CytD) [5 μg/ml] (Calbiochem, La Jolla, CA) was added to APC cultures (319). The treatment markedly inhibited the internalization of CM-DiI-labeled bacteria (<4% of the MFI uptake by untreated cells).

Fixation of BMM. Following co-culture with Pn14, and extensive washing to remove free Pn14, BMM were fixed by incubation for 30 min in 1% paraformaldehyde in

PBS at RT. Cells were washed again 3x, and the cell count was determined before i.v. injection

Statistics. Data were expressed as geometric mean ± S.E.M. of the individual results. Student's t-test was used to determine statistical significance between groups. $P<0.05$ was considered statistically significant.

Results

Phenotypic characterization of unstimulated and Pn14-activated BMDC, BMM, and PerM populations in vitro. We previously demonstrated that BMDC pulsed with intact heat-killed Pn14 elicited, via distinct mechanisms, both protein- and PS-specific Ig isotype responses upon adoptive transfer into naïve mice (43). Although DC, in contrast to macrophages, are thought to be critical for initiating humoral immune responses in naive mice, a direct demonstration of this in vivo is still largely lacking. We thus wished to compare the ability of Pn14-pulsed macrophages and DC to elicit antigen-specific Ig isotype responses in this adoptive transfer model. Two distinct macrophage populations were utilized: freshly-harvested peritoneal macrophages (PerM) obtained in the absence of thioglycolate elicitation, and macrophages obtained after culturing bone marrow cells for 6 days in M-CSF (BMM). A similar approach, in which BM cells were instead cultured in GM-CSF, was used to produce BMDC. BMDC were only lightly adherent to the tissue culture plastic, and were easily removed by pipetting (320). In contrast, BMM and PerM were firmly adherent and were detached using lidocaine,

instead of the more traditional method of cell scraping, since we found the latter caused significant cell death (data not shown).

The 3 cell populations cultured in medium alone each exhibited distinct patterns of expression of a number of key cell surface phenotypic markers (Figure 10). As expected (117, 321), BMM and PerM both expressed, in a comparable fashion, significantly higher levels of F4/80, CD11b and CD14 than BMDC. Whereas all 3 populations expressed CD11c, BMM surprisingly exhibited the highest expression. Of interest, whereas BMDC broadly expressed a high mean level of MHC-II, compared to the uniformly low expression by BMM, a major sub-population of PerM constitutively exhibited high levels of MHC-II. In contrast, all 3 populations expressed low levels of CD40, although PerM exhibited somewhat higher levels than either BMDC or BMM. CD86 expression on unstimulated BMDC was low, whereas BMM and PerM exhibited moderately higher and comparable levels. In contrast, significant CD80 expression was observed on both BMDC and BMM, but found at much lower levels on PerM. These data confirm two distinct populations of macrophages that in turn each contrast with BMDC.

Following 24h of culture with intact heat-killed Pn14, expression of MHC-II, CD80, CD86, CD40, CD11b, and CD11c each decreased significantly on viable PerM relative to cells cultured in medium alone (Figure 10). In contrast, CD40 expression was markedly upregulated on both BMDC and BMM following Pn14-mediated activation, whereas each moderately increased CD86. No significant modulation of either MHC-II or CD80 was observed in BMM, whereas BMDC exhibited a moderate increase in CD80, although no significant change in mean MHC-II expression. BMDC also upregulated CD11c. Collectively, the phenotypic profiles of both unstimulated PerM and Pn14-

activated BMDC suggest that these cells may, during some time interval, be potentially effective APC for CD4+ T cells. However, low expression of MHC-II on BMM, in the absence or presence of Pn14, despite relatively high expression of costimulatory molecules following activation, suggest that these cells are likely to function poorly as APCs.

BMDC produce higher levels of pro- and anti-inflammatory cytokines than BMM or PerM in response to Pn14. Secretion of cytokines by activated APC can significantly impact on CD4+ T cell activation, and differentiation into distinct subsets, with subsequent differing effects on humoral immunity (322). Specifically, we previously demonstrated that early release of endogenous pro-inflammatory cytokines (i.e. TNF-α, IL-6, IL-12, and IFN-γ) stimulate the antigen-specific Ig response to intact Pn, whereas the anti-inflammatory cytokine IL-10 is inhibitory (38). Alternatively, we also observed that autocrine IL-10 can delay the onset of apoptosis of Pn-activated DC, resulting in enhanced humoral responses (323). In this regard, we wished to compare the ability of BMDC, BMM, and PerM to secrete IL-6, TNF-α, IL-12, and IL-10 in vitro in response to graded doses of Pn14. As illustrated in Figure 11A, BMDC secreted significantly higher levels of all 4 cytokines than BMM or PerM, especially at the higher doses of Pn14. BMM made higher levels of IL-12 than PerM, whereas the latter was more sensitive to low dose Pn14 for TNF-a secretion. We previously demonstrated that Pn14 elicits cytokine production by splenic macrophages and DC through the combined action of TLR2, TLR4, and TLR9 (63). In this regard, a similar pattern of IL-12 secretion as that observed for intact Pn14, was also observed using optimal amounts of individual TLR

ligands (i.e. LPS [TLR4], CpG-ODN [TLR9], and Pam_3Cys [TLR2]) (324) (Figure 11B). Likewise, BMDC secreted higher amounts of TNF-α than BMM or PerM in response to all 3 TLR ligands, and PerM made more TNF-α than BMM at least in response to LPS. Although these data indicate that BMDC elicit a more robust in vitro cytokine response to Pn14 than either BMM or PerM, it is not clear whether the higher levels of IL-10 secreted by BMDC would counteract the potential in vivo Ig-stimulatory effects of the enhanced IL-6 and TNF-α response.

BMM>BMDC>PerM for phagocytosis of Pn14. Although both macrophages and DC can phagocytose intact bacteria, the rate by which this occurs could impact on their ability to act as APC. In particular, we were interested in establishing conditions to obtain roughly comparable bacterial uptake by BMDC, BMM, and PerM in vitro, in order to better compare their ability to induce humoral responses upon adoptive transfer into naïve mice. In a first set of experiments, Pn14 was labeled with the fluorescent dye CM-Dil and added to cultures of APC at a ratio of 800 CFU Pn14/APC for varying periods of time. The mean fluorescence intensity of the 3 APC populations was measured to compare the relative uptake of bacteria at each time point. Since fluorescence could reflect cell surface binding rather than actual uptake, we included control groups consisting of the various APC cultured with Pn14 in the presence of cytochalasin D, an inhibitor of microfilament assembly, and hence phagocytosis, for 300 min (the latest time point). We previously demonstrated that cytochalasin D blocks Pn14 uptake into BMDC. As illustrated in Figure 12A the efficiency of uptake of Pn14 was BMM>BMDC>PerM. With the addition of concentrations of Pn14 >800 CFU/APC these differences could be

somewhat narrowed over time (Figure 12B). In a separate study we determined that BMDC, BMM, and PerM internalized an average of 27, 58, and 14 CFU of Pn14 per cell, respectively following 4h of culture with 2,000 CFU Pn14/APC. We used these conditions for our subsequent adoptive transfer experiments.

Pn14-pulsed BMM and PerM elicit humoral immune responses comparable to BMDC upon adoptive transfer into naïve mice. BMDC, BMM, and PerM were pulsed with Pn14 in vitro under the conditions cited above. After thorough washing to remove free bacteria, 1 x 10^6 Pn14-pulsed APC were injected i.v. per mouse. As controls, free Pn14 and unpulsed APC were also injected i.v. into separate groups of mice. The percentages of Pn14-pulsed BMDC and BMM found in the spleen, 24h after i.v. injection were 0.01% and 0.03% of the total spleen cell population, respectively (significant, $p<0.05$). Fourteen days post-immunization, all groups were boosted i.p. with free Pn14 to asses the potential generation of memory during the primary immunization. Sera were collected on days 0 (pre-bleed), 7, 14 (primary), and 21 (secondary) for determination of titers of IgG specific for pneumococcal surface protein A (PspA), and IgM and IgG specific for the type 14 capsular polysaccharide (PPS14). As illustrated in Figure 13A, Pn14-pulsed BMDC, BMM, and PerM each effectively primed naïve mice, in a comparable manner, for a secondary IgG anti-PspA response following boosting, on day 14, with free Pn14. Thus, day 21 serum titers of IgG anti-PspA were significantly ($p<0.05$) higher in all groups primed with Pn14-pulsed APC relative to mice primed with unpulsed APC, and relative to naïve mice, 7 days after immunization with free Pn14.

Additionally, a small, but significant, primary IgG anti-PspA response was also elicited by all Pn14-pulsed APC, in contrast to undetectable titers in mice primed with unpulsed APC (Figure 13A). In contrast, none of the Pn14-pulsed APC populations primed mice for a secondary IgM or IgG anti-PPS14 response following boosting with free Pn14 (Figure 13A). However, all Pn14-pulsed APC induced substantial primary IgM and IgG anti-PPS14 responses, in contrast to the undetectable titers observed in mice injected with unpulsed APC. Additionally, no significant differences in day 21 serum titers of PspA-specific IgG3, IgG1, IgG2b, or IgG2a were observed between any of the groups primed with Pn14-pulsed APC and boosted with free Pn14 (Figure 13B). With the exception of significantly higher day 14 serum tiers of PPS14-specific IgG3 and IgG1 in mice injected with Pn14-pulsed BMM relative to BMDC, PPS14-specific IgG isotypes were otherwise comparable among the 3 groups (Figure 13B).

Pn14-pulsed BMM play an active role as APC for initiating the PspA-specific IgG response. The ability of Pn14-pulsed macrophages to initiate a humoral immune response upon adoptive transfer into naïve mice could reflect an active process mediated by the transferred macrophages or, alternatively, the passive transfer by macrophages of intact Pn14 and/or Pn14-derived antigens to other immune cell types. Further, Pn14-pulsed macrophages could be playing a direct and critical role in presenting Pn14-derived proteins to CD4+ T cells in vivo or, alternatively, might be actively transferring Pn14 proteins to endogenous APC. In the following series of studies to test these possibilities, we focused solely upon BMM, as opposed to PerM, because of our ability to obtain substantially larger numbers of BMM for our experiments. BMM were pulsed with Pn14

as described above for adoptive transfer (see Figure 13), then either fixed with paraformaldehyde or left untreated, immediately prior to i.v. injection. The percentages of Pn14-pulsed viable and fixed BMM found in the spleen, 24h after i.v. injection were 0.02% and 0.06% of the total spleen cell population, respectively (significant, $p<0.05$). As illustrated in Figure 14 viable, but not fixed, Pn14-pulsed BMM elicited a primary PspA-specific IgG response, and primed mice for a secondary IgG response following boosting with free Pn14, similar to that observed in Figure 13. In distinct contrast, fixed Pn14-pulsed BMM elicited an IgG anti-PPS14 response that was not significantly different than that for viable BMM, whereas the IgM anti-PPS14 was only modestly, though, significantly reduced (Figure 14). Thus, viable BMM are required in vivo for induction of the PspA-, though not PPS14-specific, Ig response.

To assess whether transferred Pn14-pulsed BMM were playing an active role as APC in vivo for induction of the PspA-specific IgG response, we first wished to determine whether this response required endogenous T cells. We previously demonstrated that both the IgG anti-PspA and IgG anti-PPS14 response to free Pn14 was dependent on CD4+, but not CD8+, T cells, whereas the IgM anti-PPS14 response was T cell-independent (217, 218) Pn14-pulsed BMM or free Pn14 were injected into either naïve wild-type or athymic nude mice, the latter being markedly deficient in T cells. As illustrated in Figure 15 both Pn14-pulsed BMM and free Pn14 significantly ($p<0.05$) primed wild-type mice, but not athymic nude mice, for a subsequent secondary IgG anti-PspA response following boosting with free Pn14. In distinct contrast Pn14-pulsed BMM elicited an equivalent primary IgG anti-PPS14 response in wild-type and athymic nude mice (Figure 15). Boosting of wild-type mice, initially injected with Pn14-pulsed BMM,

with free Pn14 increased the IgG anti-PPS14 response only to those levels seen in wild-type mice immunized with free Pn14 alone, confirming that there was no significant priming by Pn14-pulsed BMM for induction of PPS14-specific IgG. These data thus demonstrate that the IgG anti-PspA response to Pn14-pulsed BMM is dependent on endogenous CD4+ T cells.

We next wished to address whether the IgG anti-PspA response to Pn14-pulsed BMM required cognate interactions between the transferred BMM and the endogenous CD4+ T cells. Thus, BMM were obtained from mice genetically deficient in MHC-II. Wild-type and MHC-II-/- BMM were pulsed with Pn14 in vitro and injected i.v. into naïve wild-type mice followed by boosting i.p. with free Pn14 on day 14. A separate group of wild-type mice were immunized and boosted with free Pn14 alone as a control. In contrast to wild-type Pn14-pulsed BMM, MHC-II-/- BMM exhibited a complete failure to prime for a secondary IgG anti-PspA response (Figure 16A). In contrast, both wild-type and MHC-II-/- Pn14-pulsed BMM elicited equivalent IgM and IgG anti-PPS14 responses. In a second, analogous set of studies, we compared wild-type and CD40-/- Pn14-pulsed BMM for their ability to elicit Ig responses upon adoptive transfer into wild-type mice. During cognate interactions, CD40-ligand on activated T cells binds CD40 expressed by APC, resulting in delivery of a potent costimulus for cytokine secretion and phenotypic maturation of the APC. In this regard, we observed that the primary IgG anti-PspA response elicited by Pn14-pulsed CD40-/- BMM was markedly reduced relative to wild-type BMM, whereas priming by CD40-/- BMM for the secondary response was partially, though significantly, reduced as well (Figure 16B). In contrast, CD40-/- and

wild-type BMM elicited essentially equivalent IgM and IgG anti-PPS14 responses (Figure 16B).

BMDC pulsed with either recombinant PspA or intact Pn14 are more potent stimulators of IL-2 secretion by a PspA-specific T hybridoma, than similarly treated BMM. Collectively, the data so far strongly suggest that Pn14-pulsed BMM, in addition to BMDC, can play an active role as APC for CD4+ T cells when adoptively transferred into naïve mice. In a final set of experiments we therefore wished to assess the relative ability of BMDC and BMM to act as APC for CD4+ T cells upon uptake of Pn14. We thus created an MHC-II^{d}-restricted CD4+ T hybridoma (BALD4) specific for the Pn14 cell wall protein, pneumococcal surface protein A (PspA) [see Methods]. In preliminary studies we observed that BMDC cultured with Pn14 failed to stimulate BALD4 for IL-2 secretion (data not shown). This was in distinct contrast with either recombinant PspA, intact Pn, capsular type 2 (strain D39) or its unencapsulated isogenic mutant (strain R36A). In light of previous reports that different strains of Pn can express antigenically-distinct families/seroclades of PspA, we concluded that BALD4 recognized an epitope of PspA not expressed by our Pn14 strain. We thus genetically engineered a new strain of capsular type 14 Pn (R6-14) that produced the same family 1/seroclade 2 of PspA as strain R36A (both recognized by the mAbs DC10-IA5 and CF9IIB7, and able to stimulate BALD4), but expressed the same serotype 14 capsular PS as Pn14 (both recognized by the mAb 44.1) [see "Methods"].

BMDC and BMM were co-cultured with BALD4 for 24h in the presence of varying concentrations of PspA (0.001-25 μg/ml) or R6-Pn14 or its unencapsulated isogenic mutant (R6) [0.03-67 x 10^6 CFU/ml]. At the end of the culture period, SN was collected for determination of IL-2 concentrations by ELISA. In 3 independent experiments BMDC were found to be 12 +/- 2 (arithmetic mean +/- SEM)-fold more efficient at presenting PspA than BMM (significance $p<0.05$) (Figure 17). This efficiency reflects the amount of additional PspA required for BMM to give an IL-2 response equivalent to that induced by BMDC. The difference in efficiency between BMDC and BMM co-cultured with either R6-14 or R6, for induction of IL-2 by BALD4 was even more marked than that observed for PspA: 443 +/- 34 for R6-14 (2 independent experiments) and 418 +/- 117 for R6 (3 independent experiments) (Figure 17). Of interest, although it has been suggested that the capsular PS might interfere with, or otherwise modulate, the presentation of bacterial protein to T cells (325-328) the IL-2 induction profiles using R6-14 and R6 were essentially identical. The graphs in Figure 17 were aligned so that the amount of PspA, calculated by an inhibition ELISA assay, contained in a given dose of R6-14 or R6 is directly (vertically) beneath the concentration given for PspA in the uppermost graph. This indicates that BMDC present PspA ~100-fold more efficiently to BALD4 when expressed by the intact bacteria as opposed to being present in an isolated, soluble form (Figure 17). Collectively, these data strongly suggest that Pn14-pulsed BMM can play an active role as APC for eliciting a TD humoral immune response in vivo, comparable to that observed for Pn14-pulsed BMDC, despite a markedly lower APC efficiency in vitro to stimulate a PspA specific hybridoma relative to BMDC.

Discussion

Although many studies have documented the ability of macrophages to internalize pathogen-associated antigens, and present them to *pre-primed* T cells (329-333), it is widely assumed that the induction of CD4+ T cell-dependent antibody responses in *naïve* mice is uniquely dependent upon initial antigen presentation by DC (221, 222). Following this event, antigen-specific B cells engage in cognate interactions with DC-primed CD4+ T cells to elicit effector functions critical for induction of Ig secretion and class switching (334). Although a role for macrophages as APC in this latter process is largely obscure, macrophages may regulate humoral immunity through other mechanisms, including transfer of intact antigen to B cells (335, 336), secretion of BAFF (337) and/or local release of C3 (42). Although the indirect evidence in support of a central role for DC as APC in naïve mice is extensive, there is to our knowledge, no published data directly demonstrating that endogenous DC are indeed indispensable for eliciting in vivo humoral immune responses. Thus, although several studies, using CD11c-DTR Tg mice, treated with DT to deplete DC, demonstrated a critical role for endogenous DC in eliciting a CD8+ CTL response (309-311) or CD4+ T cell priming (312) in the model systems studied, no comparable data on humoral immunity have as yet

been reported. Further, although a number of imaging studies have documented early DC-CD4+ T cell interactions in situ, following immunization with protein antigen (313), there is little indication that potential macrophage-CD4+ T cells were extensively investigated, especially in the spleen, leaving open their possible role as initiating APC for naïve T cells.

Two groups studied the ability of antigen-pulsed macrophages to elicit immunity upon adoptive transfer into naïve mice, and surprisingly both reported that they were indeed capable of acting in a manner comparable to that of antigen-pulsed DC. In the series of studies by Moser and colleagues (223, 305-307) macrophages pulsed in vitro with protein antigen were shown to elicit specific antibody production upon injection into naïve mice. However, the possibility that the injected macrophages were acting largely via passive transfer of antigen to endogenous DC was not ruled out. However, a study by Pozzi et al, demonstrated the ability of peptide-pulsed macrophages to elicit CD8+ CTL responses upon injection into naïve mice, in which a role for endogenous APC in the recipients was clearly ruled out (308).

In our current study we demonstrate that Pn14-pulsed macrophages play an active role in eliciting the IgG anti-PspA response, upon their transfer into naïve mice. This response was abrogated in T cell-deficient recipients, consistent with our earlier studies using free Pn14, in which an absolute dependence on CD4+ T cells was demonstrated (217). We further show that the induction of the IgG anti-PspA response by Pn14-pulsed macrophages was not due exclusively to passive transfer of PspA to endogenous APC. Thus, macrophages from mice genetically deficient in MHC-II or CD40, or wild-type macrophages made non-viable prior to transfer, were defective in eliciting the IgG anti-

PspA response in wild-type mice, following in vitro pulsing with intact Pn14. This requirement for macrophage expression of MHC-II and CD40 further supports the notion that these cells were acting as APC for CD4+ T cells. We believe that it is highly unlikely that the observed Ig-inducing effects of the Pn14-pulsed macrophage populations used in this study were due to contaminating DC. Firstly, we utilized cells that were firmly adherent to the tissue culture plastic following 7 days of culture in the presence of M-CSF-1, a feature not traditionally observed for DC. Secondly, phenotypic analysis of the macrophage populations revealed an essentially unimodal population of cells with a relatively high expression of F4/80, CD14, and CD11b, characteristic of macrophages (117, 321). Finally, the ability of the Pn-pulsed BMM population to present PspA to a PspA-specific T hybridoma was >400-fold less efficient than Pn-pulsed BMDC.

Our data do not formally rule out the possibility that injected macrophages released PspA to endogenous DC in the recipient mice, and subsequently primed naïve CD4+ T cells. The role of the macrophage in this potential scenario could have been to actively promote effector function of the DC-primed T cells, through MHC-II- and CD40-dependent cognate interaction, which was then critical for induction of the IgG anti-PspA response. We do not favor this latter possibility, however, for at least two reasons. Firstly, several studies have demonstrated a lack of transfer of antigen between macrophages and DC when studying T cell activation (301, 338). Secondly, we previously demonstrated that DC pulsed with Pn14 in vitro could also elicit an IgG anti-PspA response upon adoptive transfer into naïve, wild-type mice, which critically depended upon expression of MHC-II, CD40, and B7, as well as viability, of the injected

DC (43). These data indicated that endogenous DC, in this system, were not capable of priming the mice via potential uptake of PspA released by the injected DC.

We demonstrate that Pn14-pulsed macrophages can stimulate a PspA-specific T hybridoma in an MHC-II-restricted manner following uptake of intact Pn14 or free PspA, although with much lower efficiency than DC. Others, using naïve or primed CD4+ T cells have also demonstrated a similar difference in APC efficiency (221, 222). Our data is somewhat unique in that we directly compared presentation of a protein expressed by an intact extracellular bacterium with the same protein, produced in an isolated, soluble form. In this regard, we show a much greater difference in APC efficiency between DC and macrophages when using intact Pn, as opposed to soluble PspA. The reason for this difference is unclear, although soluble and particulate antigens are known to be internalized by, and traffic differently within APC (339, 340). Of note, although it has been suggested that polysaccharides might interfere with, or otherwise regulate, APC presentation of associated proteins (325-328) we find no difference in APC efficiency when using an encapsulated Pn vs. its unencapsulated, isogenic variant.

Collectively, the documented ability of DC to act as superior APC for naïve CD4+ T cells, would appear to argue against a role for macrophages as initiators of humoral immunity. However, this could be an oversimplification, especially in regard to Ig responses induced by intact bacteria. Thus, the differences observed in APC efficiency between macrophages and DC are relative, not absolute. The threshold level for initial priming of naïve CD4+ T cells required to initiate an in vivo humoral immune response is not clear. Further, macrophages are uniquely positioned in secondary lymphoid organs, and functionally specialized, to efficiently internalize incoming bacteria (117, 341). In

this regard, we observed that BMM were more efficient than BMDC in uptake of Pn14. Large numbers of macrophages may also be recruited into secondary lymphoid organs such as the spleen, especially in response to a systemic bacterial infection. Macrophages present in an inflammatory milieu will likely upregulate cell surface costimulatory molecules and release cytokines that may enhance naïve CD4+ T cell priming. We demonstrate that in response to Pn14, which contain multiple TLR ligands, macrophages upregulate CD40 and CD86, and release pro-inflammatory cytokines, including the T_H1-inducing cytokine, IL-12 (342). Of interest, it was previously demonstrated that macrophages pulsed in vitro with soluble protein in the absence of a TLR ligand, elicit a predominant Th2 antibody response upon adoptive transfer (223, 306), whereas additional stimulation with a TLR ligand resulted in the induction of both Th1 and Th2 IgG isotypes, similar to DC pulsed with antigen in the absence of TLR stimulation (305). In this regard, we demonstrate that Pn14-pulsed DC and macrophages also elicited a similar PspA-specific IgG isotype profile upon transfer in vivo, and subsequent boosting with free Pn14. Macrophages are also functionally heterogeneous (118, 341, 343), even in regard to their ability to act as APC. In particular, one study demonstrated that 20% of splenic macrophage precursors could process and present a soluble protein antigen to naïve CD4+ T cells, that was positively correlated with their ability to secrete IL-12 (300). Thus, a particular macrophage subset, used in studies of APC function, may not accurately reflect the overall APC potential of the mixed macrophage population in vivo, especially within an inflammatory milieu. Of interest, although in our study BMM and PerM differed in their phenotypic profile (in the absence and presence of Pn14), level of

cytokine secretion, and rate of Pn14 internalization, they generated roughly similar in vivo Ig responses upon adoptive transfer.

In contrast to the in vivo IgG anti-PspA response induced by Pn14-pulsed BMM, the IgG anti-PPS14 response was not dependent on BMM expression of MHC-II or CD40, was largely independent of BMM viability immediately prior to transfer, and was T cell-independent. However, as previously described (217), the IgG anti-PPS14 response to free Pn14 was T cell-dependent. The IgG anti-PPS14 response elicited by Pn14-pulsed BMM also differed in several respects with what we previously observed using Pn14-pulsed BMDC (43). Using BMDC, the IgG anti-PPS14 response did dependent on BMDC viability and was T cell-dependent, although with the exception of the IgG1 isotype, not dependent on BMDC expression of MHC-II or CD40. The mechanism by which Pn14-pulsed BMM elicited the anti-PPS14 response is not clear, although macrophages have been previously implicated in stimulating T cell-independent antibody responses. Thus, it was demonstrated that macrophages can directly enhance proliferation of B cells in vitro (337). Further, in vitro T cell-independent B cell responses to TNP-Ficoll, TNP-LPS and TNP-*Brucella abortus* are dependent on the presence of macrophages (344-347). B cells that are stimulated by TNP-Ficoll-pulsed macrophages appear to be a distinct B cell subset that is uniquely responsive to macrophage signals (348). Hence, direct activation of B cells by macrophages, even perhaps macrophages in a non-viable state, might account for their inductive effect. Alternatively, macrophages could have released free Pn14 or PPS14 following adoptive transfer, and directly stimulated the endogenous immune system of the recipient.

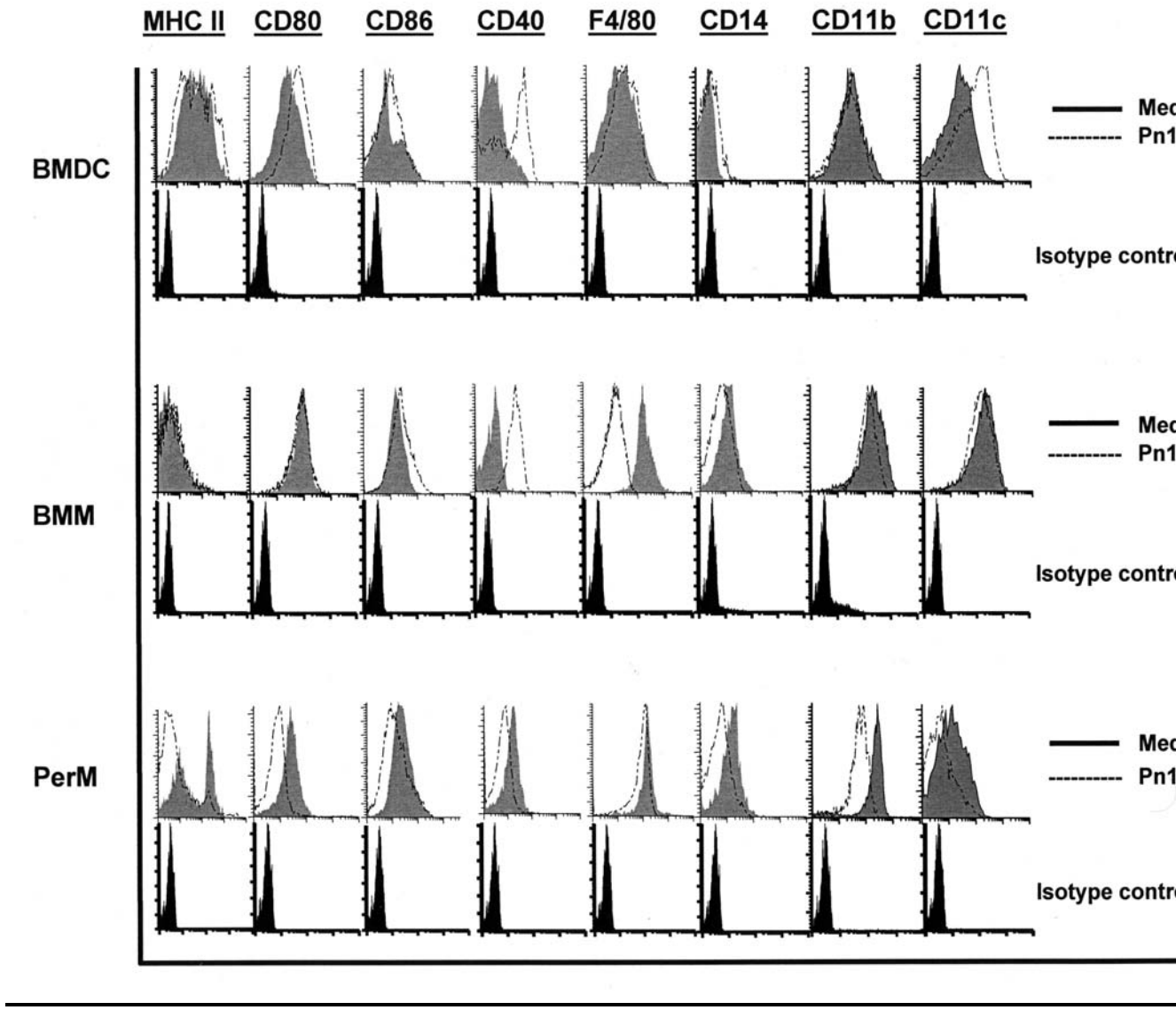


Figure 10. Cell surface phenotype of unstimulated and Pn14-activated BMDC, BMM, and PerM.

Bone marrow cells from BALB/c mice, cultured initially for 7 days in the presence of either GM-CSF (BMDC) or M-CSF (BMM), or freshly-explanted, non-elicited PerM were stained with fluorochrome-labelled mAbs specific for the indicated cell surface proteins after a 24h culture period (1 x 10^6 cells/ml) in medium alone (filled histogram) or in the presence (open histogram) of Pn14 (2 x 10^9 CFU/ml), and analyzed by flow cytometry. Results representative of 2 independent experiments

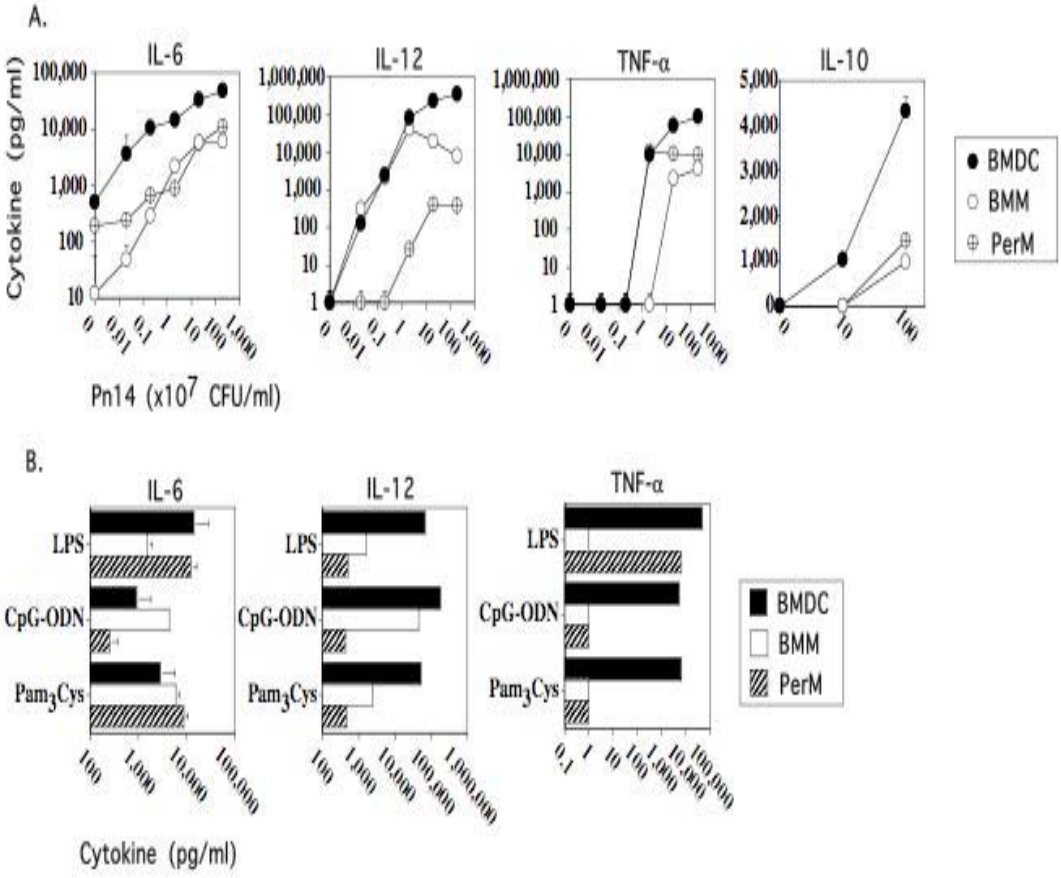

A.
IL-6
IL-12
TNF-α
IL-10
Cytokine (pg/ml)
Pn14 (x10^7 CFU/ml)
BMDC
BMM
PerM
B.
IL-6
IL-12
TNF-α
LPS
CpG-ODN
Pam3Cys
Cytokine (pg/ml)
BMDC
BMM
PerM

Figure 11. Cytokine secretion by BMDC, BMM, or PerM in the absence or presence of Pn14 or TLR ligands.

BMDC, BMM and PerM obtained from BALB/c mice were cultured (1 x 10^6 cells/ml) for 24 h in medium alone or in the presence of (A) varying concentrations of Pn14 or (B) LPS (2 μg/ml), CpG-ODN (2 μg/ml), or Pam_3CSK4 (150 ng/ml). Concentrations of cytokines in the culture SN were measured by ELISA. Values represent the geometric mean ± SEM. Representative of 2 independent experiments.

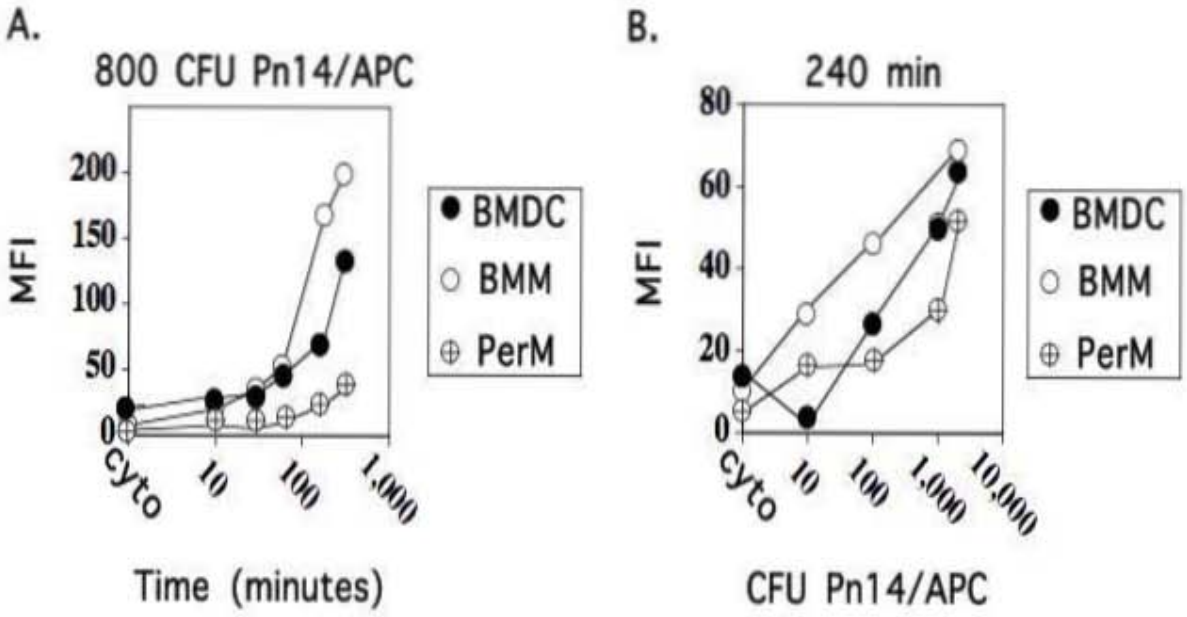

A.
800 CFU Pn14/APC
MFI
200
150
100
50
0
cyto
10
100
1,000
Time (minutes)
BMDC
BMM
PerM
B.
240 min
MFI
80
60
40
20
0
cyto
10
100
1,000
10,000
CFU Pn14/APC
BMDC
BMM
PerM

Figure 12. Uptake of Pn14 by BMDC, BMM, and PerM.

BMDC, BMM, and PerM obtained from BALB/c mice were cultured at 1 x 10^6 cells/ml in the presence of CM-Dil-labeled Pn14. (A). APC were cultured for the indicated times with 8 x 10^8 CFU of labeled Pn14 (800 CFU/APC). (B). APC were cultured for 240 min with the indicated concentrations of labeled Pn14. Cytochalasin D ("cyto"), an inhibitor of phagocytosis, was added to selected groups to control for surface binding of labeled Pn14. APC were analyzed by flow cytometry. Results are representative of 2 independent experiments.

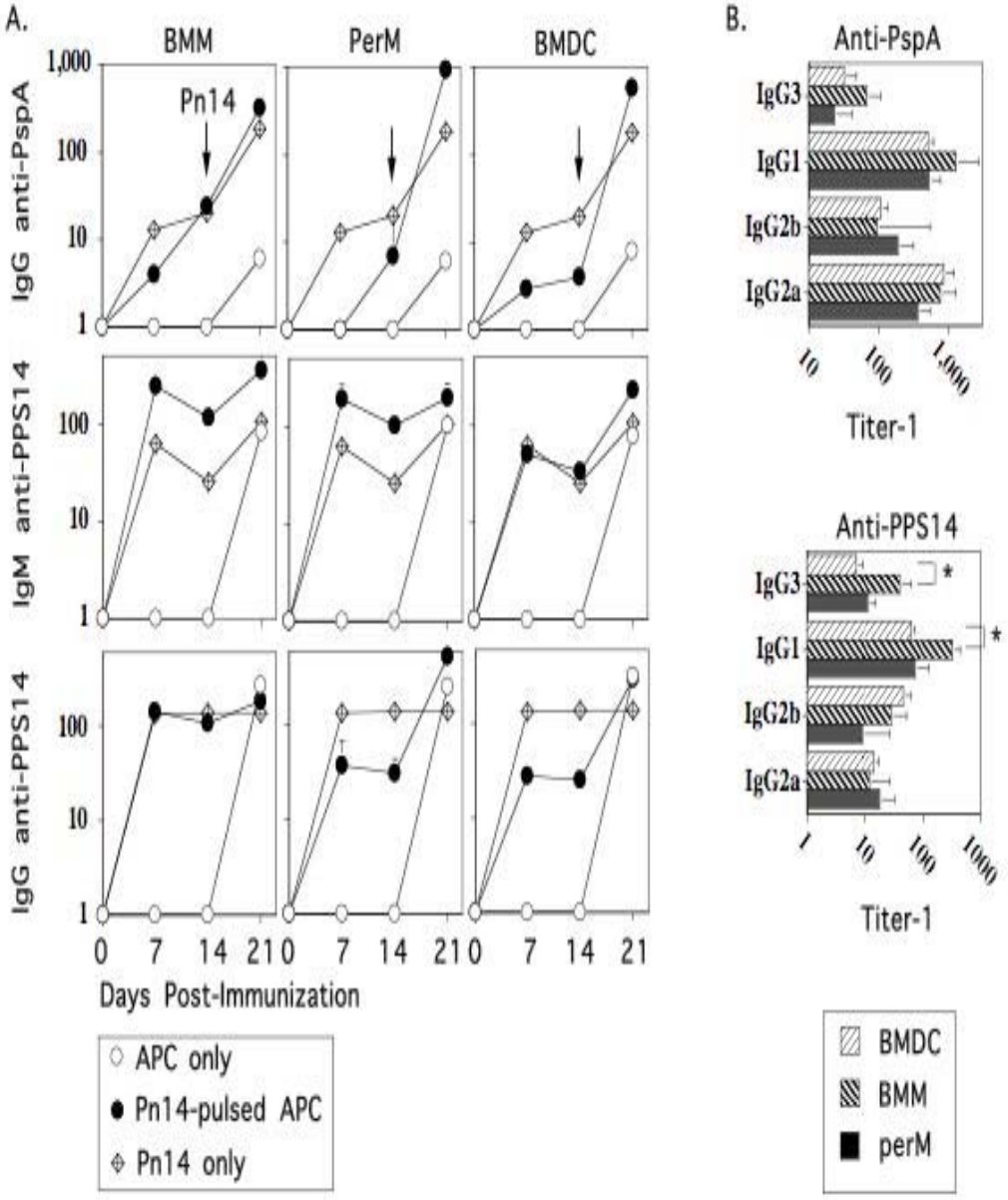

A.
BMM
PerM
BMDC
Pn14
IgG anti-PspA
IgM anti-PPS14
IgG anti-PPS14
1,000
100
10
1
0 7 14 21 0 7 14 21 0 7 14 21
Days Post-Immunization
APC only
Pn14-pulsed APC
Pn14 only
B.
Anti-PspA
IgG3
IgG1
IgG2b
IgG2a
10
100
1,000
Titer-1
Anti-PPS14
*
*
1
10
100
1000
Titer-1
BMDC
BMM
perM

Figure 13. Adoptive transfer of Pn14-pulsed BMDC, BMM, and PerM into naïve, wild-type mice.

BMDC, BMM, and PerM obtained from BALB/c mice were either cultured in medium alone or pulsed with Pn14, and injected i.v. into naïve BALB/c mice (6 mice per group, 1 x 10^6 viable APC per mouse). Mice were boosted i.p. with free Pn14 (2 x 10^8 CFU/mouse) 14 days following injection of APC. Another group of BALB/c mice (n=6) were immunized and boosted on day 14 with free Pn14 alone. Sera were collected on the indicated days for (A) measurement of titers of IgG anti-PspA, and IgM and IgG anti-PPS14 by ELISA. (B). Titers of secondary (day 21) PspA- and primary (day 14) PPS14-specific IgG isotypes were measured by ELISA from sera analyzed in "A". The data show the geometric mean ± SEM of the individual titers. Significance ($p<0.05$) is indicated in the text. Representative of 2 independent experiments.

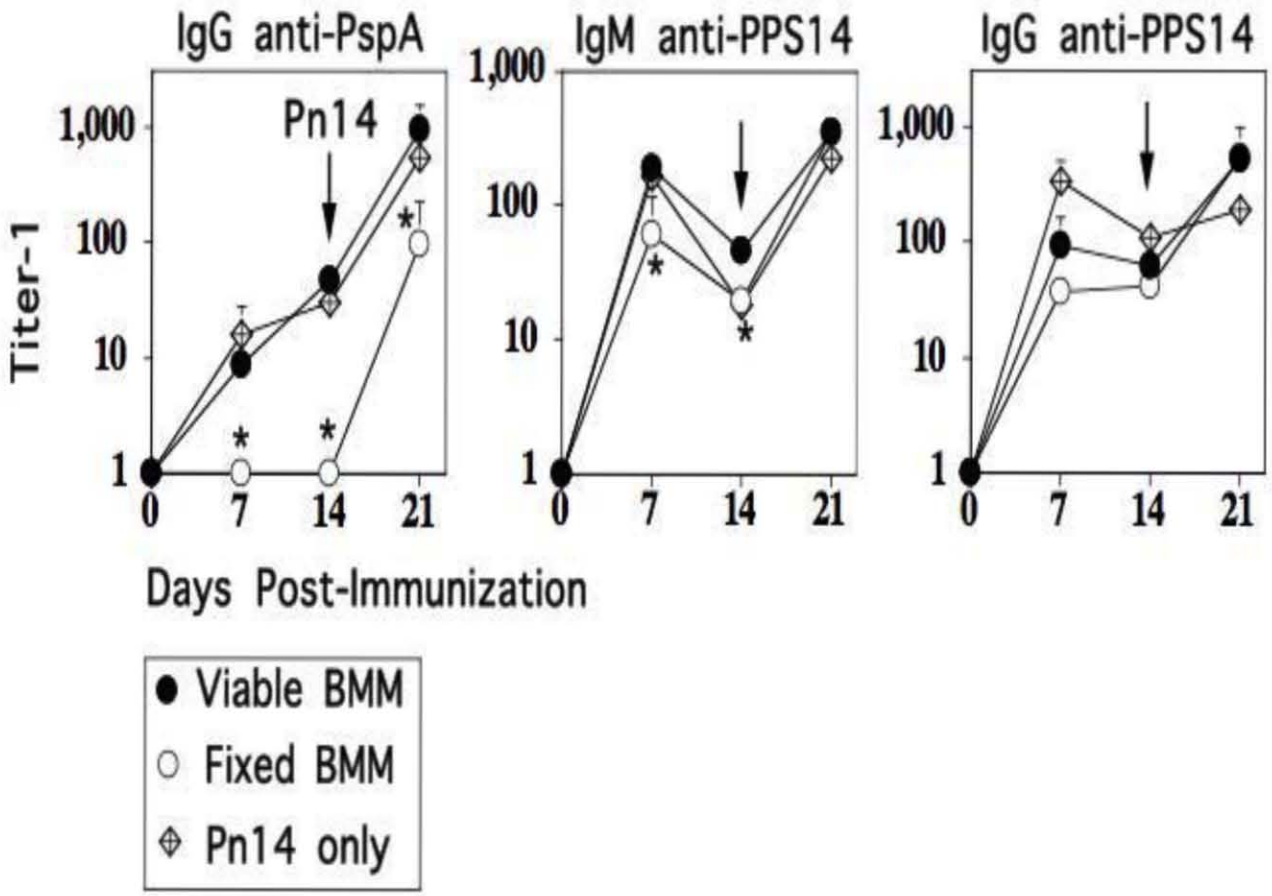

IgG anti-PspA
IgM anti-PPS14
IgG anti-PPS14
Pn14
Titer-1
1,000
100
10
1
0
7
14
21
Days Post-Immunization
Viable BMM
Fixed BMM
Pn14 only

Figure 14. Adoptive transfer of viable and paraformaldehyde-fixed Pn14-pulsed BMM.

BMM obtained from BALB/c mice were pulsed in vitro with Pn14. BMM were then either left untreated or fixed in paraformaldehyde, and immediately injected i.v. (1×10^6 APC/mouse) into naïve BALB/c mice (5 mice per group). Mice were immunized i.p. 14 days later with free Pn14 (2×10^8 CFU/mouse). A separate group of 5 mice were immunized and boosted i.p. on day 14 with free Pn14 alone (2×10^8 CFU/mouse). Sera were collected on the indicated days for measurement, by ELISA, of titers of IgG anti-PspA, and IgM and IgG anti-PPS14. The data show the geometric mean +/- SEM of the individual titers. *Significance ($p<0.05$) in comparing viable vs. fixed BMM. Representative of 2 independent experiments.

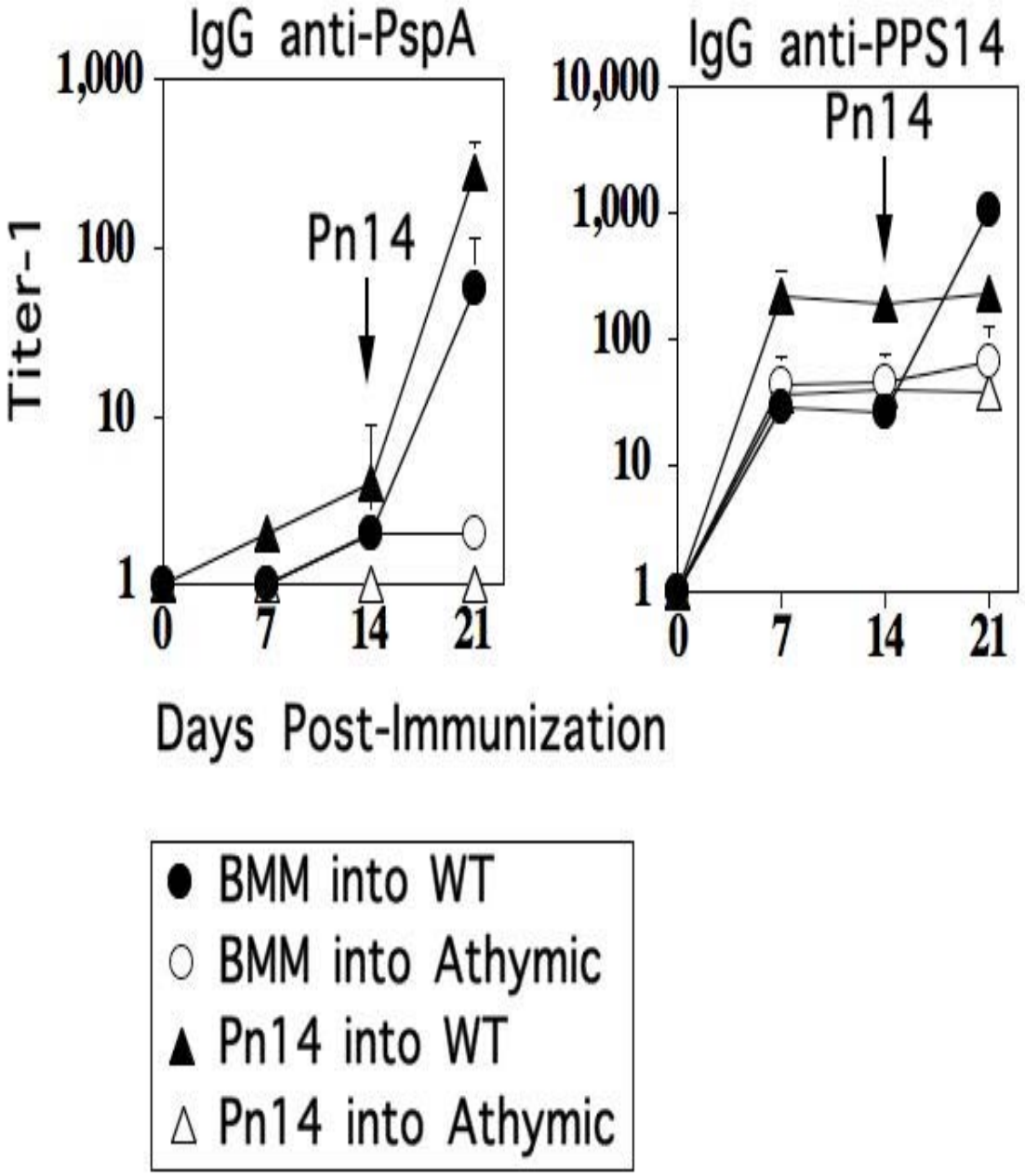

IgG anti-PspA
1,000
100
10
1
Titer-1
Pn14
0
7
14
21
IgG anti-PPS14
10,000
1,000
100
10
1
Pn14
0
7
14
21
Days Post-Immunization
BMM into WT
BMM into Athymic
Pn14 into WT
Pn14 into Athymic

Figure 15. Adoptive transfer of Pn14-pulsed BMM into athymic nude mice.

BMM obtained from BALB/c mice were pulsed in vitro with Pn14. BMM (1×10^6 APC/mouse i.v.) or free Pn14 (2×10^8 CFU/mouse i.p.) were then injected into either naïve BALB/c or athymic nude (BALB/c background) mice (5 mice per group). All groups of mice were immunized i.p. 14 days later with free Pn14 (2×10^8 CFU/mouse). Sera were collected on the indicated days for measurement, by ELISA, of titers of IgG anti-PspA or IgG anti-PPS14. The data show the geometric mean +/- SEM of the individual titers. Significance ($p<0.05$) is indicated in the text. Representative of 2 independent experiments.

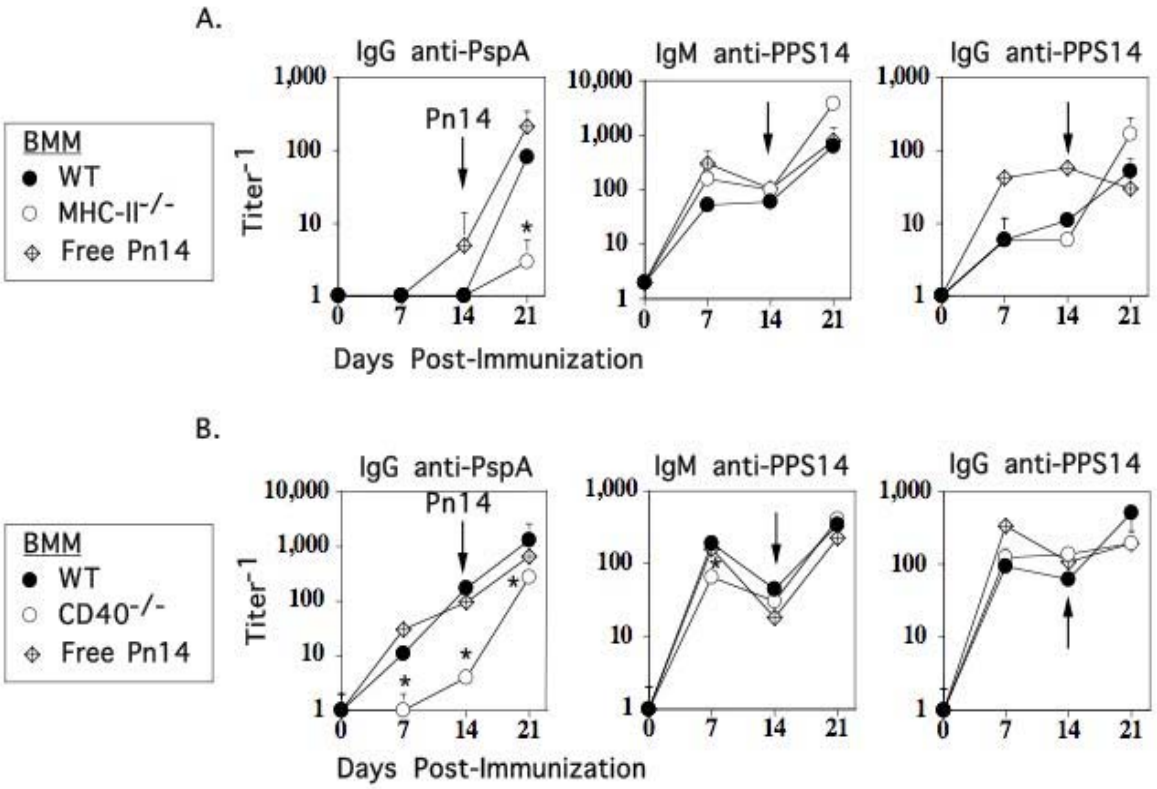

A.
IgG anti-PspA
IgM anti-PPS14
IgG anti-PPS14
Pn14
BMM
WT
MHC-II-/-
Free Pn14
Titer-1
1,000
100
10
1
10,000
0
7
14
21
Days Post-Immunization
B.
IgG anti-PspA
IgM anti-PPS14
IgG anti-PPS14
Pn14
BMM
WT
CD40-/-
Free Pn14
Titer-1
10,000
1,000
100
10
1
0
7
14
21
Days Post-Immunization

Figure 16. Adoptive transfer of Pn14-pulsed BMM from WT versus MHC-II-/- or CD40-/- mice into naïve wild-type mice.

BMM obtained from (A) C57BL/6 [WT] or MHC-II-/- mice (C567BL/6 background) or (B) BALB/c [WT] or CD40-/- mice (BALB/c background) were pulsed in vitro with Pn14. BMM (1×10^6 APC/mouse i.v.) or free Pn14 (2×10^8 CFU/mouse i.p.) were then injected into naïve C57BL/6 [A] or BALB/c [B] mice (5 mice per group). All groups of mice were immunized i.p. 14 days later with free Pn14 (2×10^8 CFU/mouse). Sera were collected on the indicated days for measurement, by ELISA, of titers of IgG anti-PspA or IgM and IgG anti-PPS14. The data show the geometric mean +/- SEM of the individual titers. *Significance ($p<0.05$) in comparing BMM obtained from WT vs knockout mice. Both "A" and "B" are each representative of 2 independent experiments.

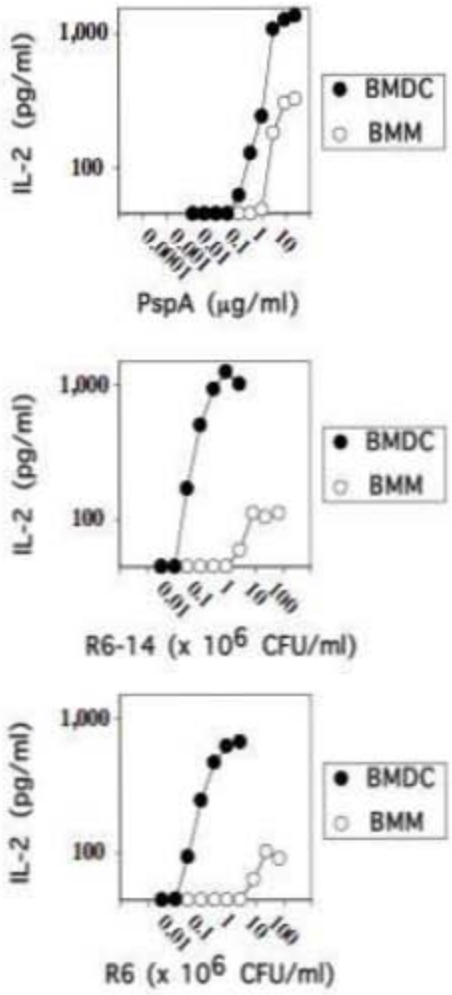

IL-2 (pg/ml)
1,000
100
BMDC
BMM
PspA (μg/ml)
R6-14 (x 10^6 CFU/ml)
R6 (x 10^6 CFU/ml)

Figure 17. APC capacity of BMDC versus BMM pulsed with either free PspA or unencapsulated or encapsulated Pn for a PspA-specific T hybridoma.

BMDC and BMM obtained from BALB/c mice were cultured for 2h at 2 x 10^4 APC/well in flat-bottom 96-well plates in the presence of the indicated concentrations of (A) PspA, (B) R6-14, or (C) R6. BALD4 (PspA-specific T hybridoma) cells were then added at 1 x 10^5 cells/well to these cultures, in the continued presence of the added antigen/bacteria, and cultured for 24h. Concentrations of IL-2 released by BALD4 cells in the culture SN were measured by ELISA. Representative of 2 independent experiments.

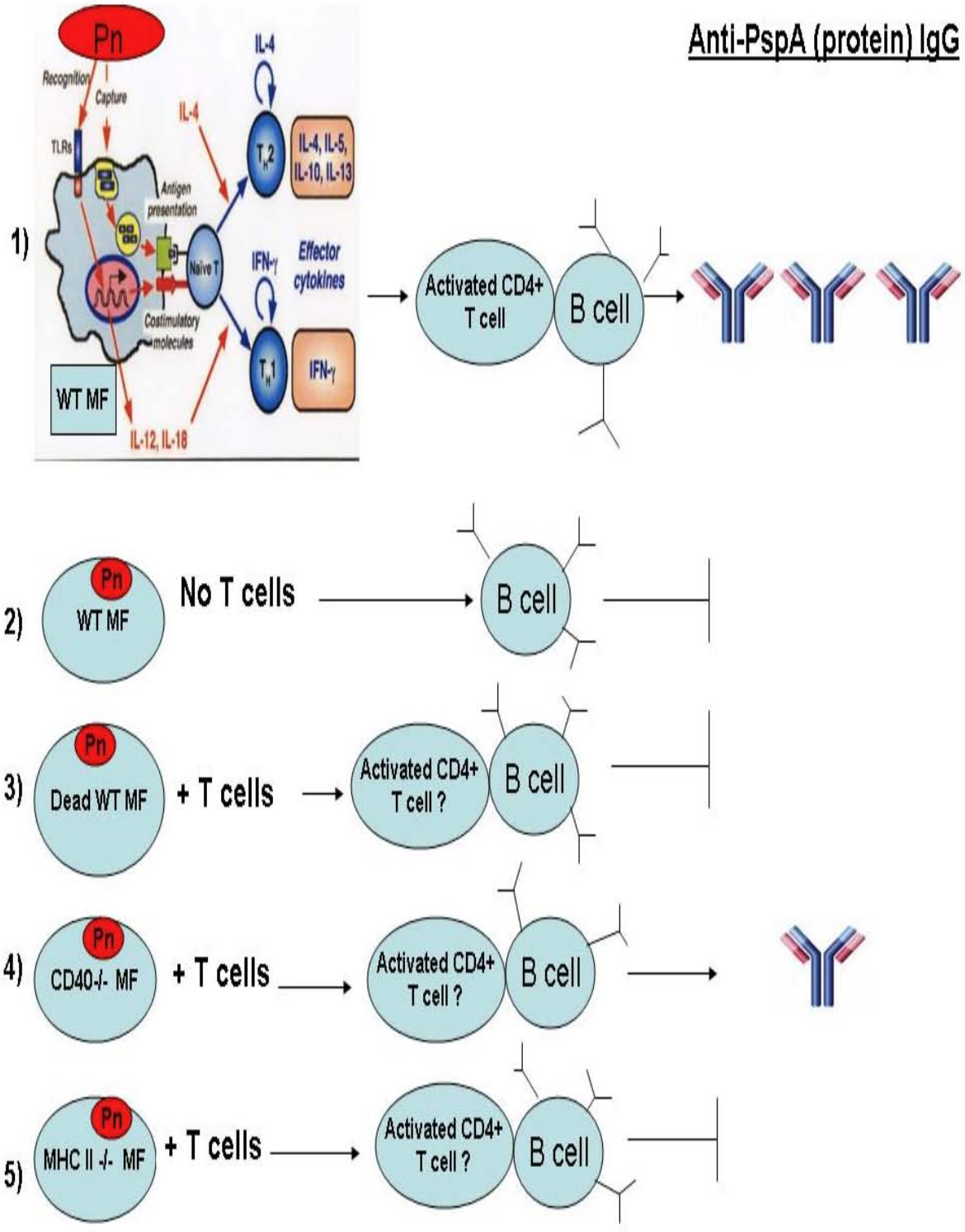

Anti-PspA (protein) IgG
1)
Pn
Recognition
Capture
TLRs
Antigen presentation
Naive T
Costimulatory molecules
WT MF
IL-12, IL-18
IL-4
IL-4
T_H2
IL-4, IL-5, IL-10, IL-13
IFN-γ
Effector cytokines
T_H1
IFN-γ
Activated CD4+ T cell
B cell
2)
Pn
WT MF
No T cells
B cell
3)
Pn
Dead WT MF
+ T cells
Activated CD4+ T cell ?
B cell
4)
Pn
CD40-/- MF
+ T cells
Activated CD4+ T cell ?
B cell
5)
Pn
MHC II -/- MF
+ T cells
Activated CD4+ T cell ?
B cell

Fig 18. Summary: Macrophages pulsed with intact *Streptococcus pneumoniae* play an active role in eliciting a T cell-dependent, pneumococcal protein-specific antibody response upon adoptive transfer into naïve mice

S. pneumonaie -pulsed MΦ are able to elicit a protein specific (anti-PspA) IgG response upon adoptive transfer into naïve mice (1). Pn- pulsed MΦ injected into T cell-deficient mice (athymic nude) were unable to elicit a PspA-specific IgG response (2) suggesting a requirement for endogenous T cells. WT naïve mice injected with MΦ that were pulsed with *S. pneumoniae* and subsequently paraformaldehyde fixed did not produce anti-PspA IgG antibodies (3) indicating MΦ play an active role as APC for initiating the PspA-specific IgG response and are not passive carriers of Ag. *S. pneumoniae*- pulsed CD40-/- and MHCII-/- MΦ that were injected into naïve WT mice elicited either an extremely defective IgG anti-PspA response (4) or did not elicit a response at all (5). Conclusion: These data indicate that *S. pneumoniae*-pulsed and adoptively transferred MΦ are able to elicit a protein- specific IgG response in a naïve mouse. This response is T-cell dependent requiring live MΦ and the expression of both MHC class II and CD40-/- on the MΦ surface.

Chapter 4

Discussion

TLR2 and its role in type I humoral immunity

Although TLRs have been shown to be important for innate immunity, there is evidence that they may play a role in adaptive immunity as well (253, 349). Our previous findings that $TLR2^{-/-}$ mice exhibit a defect in the type 1 associated (IgG2a, IgG2b, and IgG3) humoral response but an overall intact innate immune response when challenged with Pn suggested a role for TLR2 on cells involved in the adaptive immune response (e.g., DCs, T cells, and B cells). We show here that TLR2 on $CD4^+$ T and B cells, but not DC, is critical for optimal type 1 humoral immunity in response to Pn

The role of TLR2 in host defense against various pathogens has been investigated. Takeuchi et al. demonstrated that peritoneal MΦ mice deficient in My88 were not able to secrete TNF-α or IL-6, whereas $TLR2^{-/-}$ MΦ produced low but significant levels of cytokine in response to *Staphloccocus aureus*, indicating a critical role for both MyD88 and TLR2 for defense against bacterial pathogen (350). Another group using the spirochete *Borrelia burgdorferi* as model showed that infected $TLR2^{-/-}$ mice had a 10-fold higher level of infection but produced equivalent levels of IgG isotypes compared to wt mice, concluding that in response to *Borrelia burgdorferi*, TLR2 is critical for an innate but not adaptive immune response (351). Studies using the Pn meningitis model where *S. pneumoniae* were injected into the central nervous system reported that $TLR2^{-/-}$ mice had increased bacterial growth, reduced bacterial clearance, and increased inflammation compared to wt mice resulting in earlier time of death (352, 353). However two other studies, where $TLR2^{-/-}$ mice were infected with Pn intra-nasally colonizing the

nasopharynx (242, 354), showed only a modest reduction in the inflammatory response and an unaltered bacterial defense of versus wt mice. Our lab has shown that even though type 1 humoral immunity was significantly reduced, $TLR2^{-/-}$ mice were only slightly more sensitive to killing, relative to wild-type mice, after i.p. challenge with live Pn (220). Hence, depending on the model system and/ or pathogen, mice deficient in TLR2 can either exhibit an increased or no apparent sensitivity in infection. In addition TLR2 may be critical for innate or adaptive immunity, or both.

Originally TLR2 was believed to be important in the induction of T_H2 immunity. Using an experimental asthma model, Redecke *et al*. showed that activation of TLR2 by its synthetic ligand Pam3Cys elicited a T_H2 response (355). However, the current view is that TLR2 is important for a T_H1 immune response. DC pulsed with Propionebacterium acnes (Pa), a Gram-positive bacterium that induces DC maturation via TLR2 (356) were able to induce a strong Pa-specific T_H1 response when injected into wild-type mice. In $IL\text{-}12^{-/-}$ recipient mice, Pa-pulsed DCs failed to induce a Th2 response (357).

Currently, the prevailing view is that B cells require two signals for activation, clonal expansion and subsequent secretion of antigen- specific antibodies. The first signal is initiated when Ag-specific B cells acquire antigens through their B cell receptor (BCR). However this signal is insufficient for B cell activation, proliferation and antibody secretion. The second signal is provided by Ag-specific T cells that have encountered the same Ag as the B cell. These CD4+ T encounter the Ag through interaction with APC that have already migrated to secondary lymphoid organs and presented the peptides via their MHC class I and II molecules leading to T cell activation and clonal proliferation. These antigen-specific CD4+ T clones then activate the B cell

(358-361). Hence, T cell proliferation occurs before the T cell interacts with the B cell whereas, B cell proliferation and activation occurs after interactions with T cells.

Our previous reports demonstrating that endogenous release of TNF-α, IL-6 and IFN-γ independently stimulated both IgG anti-protein and anti-polysaccharide responses of various isotypes following Pn immunization and that both the anti-protein and anti-polysccharide responses were DC-dependent (43, 319) prompted us to investigate the importance of TLR2 expression on the DC. Specifically, we were interested in whether TLR2 on the DC was critical for the induction of cytokine production and the ability to undergo phenotypic maturation in response to Pn. The fact that $TLR2^{-/-}$ BMDC pulsed with Pn in vitro secrete reduced but significant amounts of cytokines relative to wild-type and were not defective in their ability to upregulate MHCII, CD80, CD86, and CD40 suggest that TLRs in addition to, or other than TLR2 may be playing an important role. Indeed, we recently demonstrated that TLR2 is synergistic with TLR4 and TLR9 in the Pn14-induced MyD88-dependent splenic cytokine and chemokine response (63). Whereas the IgM anti-PC response is T cell-independent, an optimal IgG3 anti-PC response requires TCR-$\alpha\beta^{+}CD4^{+}$ T cell help, including CD40/CD40-ligand interactions (50, 217, 218). We show that Pn14-pulsed $TLR2^{-/-}$ BMDC elicit a normal IgM and IgG3 anti-PC response when adoptively transferred into WT mice. In contrast, BCR-mediated signaling via anti-IgD dextran (αβ-dex)-activated $TLR2^{-/-}$, in contrast to WT B cells, failed to synergize with Pn14 for mitogenesis. Even more importantly, $TLR2^{-/-}$ B cells transferred into RAG-$2^{-/-}$ mice along with WT $CD4^{+}$ T cells elicited a defective IgG3, but normal IgM anti-PC response to Pn14, relative to WT B cells. Pn, by virtue of its highly repetitive polysaccharide structures expressed on its surface, can trigger B-cell activation

by cross-linking the BCR in a multivalent fashion. In addition Pn contain several TLR ligands.

There is evidence that TLR and BCR acting in concert are able to activate mature B cells (362). In addition, it is known that B cells need to go through several rounds of proliferation in order for isotype switching to occur. In this regard, a proposed mechanism for our observations is that TLR2$^{-/-}$ B cells fail to undergo the requisite number of proliferative cycles to switch adequately to IgG3. Since B cell proliferation requires T cell help, TLR2$^{-/-}$ T cells may also fail to drive B cell proliferation adequately.

There is also evidence of direct activation of B cells via TLRs. Purified CD19+ human tonsilar B cells stimulated with Pam3Cys (TLR2 ligand), R-837 (TLR7 ligand) and CpG ODN (TLR9 ligand) showed an induction of IL-6 secretion and upregulation of MHC class II in vitro (363). Borsutzky *et al.* demonstrated that a lipopeptide from *Mycoplasma fermentas* termed MALP-2 (macrophage-activating lipopetide), promoted a T cell-independent activation and maturation of follicular, B-1a, and marginal zone B cells via TLR2. MALP-2 increased the frequency of IgM and IgG secreting cells. Furthermore, these B cells upregulated receptors that are important for T cell activation and maintenance (CD19, CD25, CD80, CD86, MHC class I and II, and CD40) (364). CpG, a TLR9 ligand, has been shown to efficiently induce proliferation and secretion of antibodies by B cells in vivo and vitro (254, 365) and the TLR7 ligand ssRNA directly stimulated B cells to proliferate and secrete IgG (366). Pasare and Medzhitov (253) found that TLR4 signaling is mandatory for IgM and IgG1 production when mice were immunized with OVA-LPS or human serum albumin-LPS and that T help is required for isotype class switching. They concluded that optimal B cell responses require both T cell

help and TLR signaling. Therefore it is possible that TLR2 is not only critical for the ability of B cells to cross-talk with T cells but also is an important receptor for direct isotype switching.

It has been shown that $CD4^+$ T cells do express TLR2. Purified CD4+ cells were activated in vitro with anti-CD3ε and anti-CD28 mAb and RNA was measured for TLR 2, 3, 4, 5, and 9. Before activation naïve CD4+ T cells expressed TLR 2-5 and 9. However, after activation TLR2 and 4 were undetectable whereas TLR3 and 9 were upregulated (367). Interestingly, a study by Komai-Koma *et al.* demonstrated that naïve human $CD4^+$ T cells expressed high levels of cell-surface TLR2 after activation by anti-T cell receptor antibody CD3 and that TLR2 functioned as a costimulator receptor molecule for T cell-activation and helped to maintain T cell memory (368). Imanishi *et al.* demonstrated that only TLR2 ligands (Pam3Cys and MALP-2) and not TLR ligands 1,3,4,5,6,7, and 9, specifically induced IFN-γ production from T_H1 cells without TCR stimulation, which was markedly enhanced in the presence of IL-12. In addition Pam3Cys and MALP-2 enhanced T_H1 survival by activating CD96 and CD70. This group also showed that all of the ligands tested failed to stimulate T_H2 cells for IL-4 production (369). Therefore, TLR2 on the T cells may be critical as a costimulatory receptor for T cell activation for the naïve T cell in response to Pn14. In addition Pn may be directly activating effector functions of T cells via TLR2. .

Since we did not actually confirm if TLR2 is expressed on our naïve or activated CD4+ T cells, this could be considered for future experiments. Splenic CD4+ T cells from naïve mice would first be purified by magnetic beads and analyzed by flow cytometry for the presence of surface TLR2. To investigate if activated CD4+ T express

TLR2 on their surface the same procedure could be used for mice that have been immunized with Pn14. We also never actually confirmed the presence of TLR2 expression on our naïve or antibody- secreting B cells. Naïve B cells would first be purified from the spleen using anti-B220 magnetic beads and subsequently assayed for the presence of surface expression of TLR2 using flow cytometry. However since a large percentage of plasma cells reside in the bone marrow (370) and plasma cells downregulate surface expression of B220 (371-373) but do express cytoplasmic IgG (370), anti-IgG magnetic beads would be used for purification from the bone marrow for activated antibody secreting B cells. Another aspect of the role of TLR2 in humoral immunity that could be investigated would be the ability of $TLR2^{-/-}$ mice to form germinal centers. This could be performed by immunizing WT and $TLR2^{-/-}$ with Pn14, taking out the spleen, and visualizing the formation of germinal centers using confocal microscopy by double staining B cells with anti-FAS and anti-GL7 monoclonal fluorescent antibodies. Because TLR2 is critical for an optimal type I, but not type II humoral response and the absence of TLR2 does not abrogate the type I response completely, I would predict little if any defect in germinal center formation. Another experiment to confirm that TLR2 expression on the B cell is critical for an optimal type I humoral response would be to adoptively transfer WT CD4+T cells and TLR2-/- B cells into irradiated mice. This experiment could be performed by adoptively transferring either purified WT CD4+ T cells and WT B cells or WT CD4+ T and TLR2-/- B cells into irradiated WT or irradiated TLR2-/- mice 24 h after irradiation. 24 h after transfer of cells mice would be immunized with Pn14. Mice would be bled on day 7 and 14 and serum would be assayed for anti-PC IgG3 and IgM. A positive control would be non -

irradiated WT and TLR2-/- immunized with Pn14. I would expect that WT and TLR2-/- irradiated mice that were given WT CD4+ T cells and WT B cells would have a normal anti-PC IgG3 response. However WT and TLR2-/- irradiated mice that were given WT CD4+ T cells and TLR2-/- B would have a defective anti-PC IgG3 response.

Although in this thesis I have shown that TLR2 on the B cell is critical for T_H1-associated humoral response, we cannot conclude that TLR2 is the sole regulator of this response. It is possible that expression of other TLRs on the B cell may be critical in the regulation of a humoral response to *Streptococcus pneumoniae*. Because *Streptococcus pneumoniae* have been shown to stimulate TLR4 (374), TLR9 and TLR7 (54, 220) as well as TLR2, I would propose investigating whether these mice have a defective humoral response (both type 1 and type 2) after immunization with *Streptococcus pneumoniae.* The first set of experiments would include immunizing TLR9-/-, TLR4-/-, and TLR7 -/- mice and assaying for IgM, IgG1, IgG2a, IgG2b, and IgG3. The second set of experiments would include adoptive transfer of WT CD4+ T and WT B or WT CD4+ T cells and TLR4-/- or TLR9-/- or TLR7-/- purified B cells into RAG-/- mice. After 24 h, the next step would be to immunize RAG-/- mice with pneumococcus and collect serum day 7 and 14. The positive control would include WT mice immunized with free Pn14. Finally, the serum would be assayed for IgM, IgG isotypes. Another set of experiments could involve performing the same experiments mentioned above but with double knockout mice (i.e. TL2-/-x TLR9-/-, TLR2-/- x TLR4-/-, TLR2-/- xTLR7-/-, TLR9-/- x TLR4-/-, TLR9-/- x TLR7-/- etc). In fact a report by Lee et al. have shown that splenic cells from TLR2-/- x TLR9-/- and TLR2-/- x TLR4-/- mice have a substantially defect in their capacity to produce splenic cytokines after incubation with Pn14 in vitro (63).

Therefore, in light of this evidence, I would predict there is a high probability that a double knockout mouse would be partially but significantly defective in its humoral resonse after immunization with Pn14.

TLRs and APCs

Although TLR2 on the DC and MΦ (data not shown) is not critical for induction of a type 1 humoral response after encountering Pn, TLRs expressed on APCs, nonetheless do play a critical role in the activation of APC. TLRs expressed on APCs recognize PAMPs on pathogens and through production of cytokines, and activation of MHC-II along with costimulatory molecules APC prime naïve CD4+ T cells. We have shown that ligands for TLR2, TLR4, and TLR9 are able to trigger the secretion of IL-12, IL-6, TNF-α, and IL-10 by PerM, BMM and BMDC. However an intact pathogen such as Pn contains a number of structures that are recognized by TLRs. TLR2 recognizes lipoteichoic acid and peptidoglycan (69, 375) and TLR4 is known to be a receptor for the pneumococcus cytotoxin pneumolysin (374). Both of these receptors are expressed at the bacterial surface (54). In addition, unmethylated CpG DNA and ssRNA, both of which are components of Pn, may be able to bind intracellular TLRs 9 and 7/8 respectively (54, 220). In fact distinct TLR ligands have indeed been shown to act synergistically to promote release of inflammatory mediators by MΦ (61, 65, 66) and DC (62, 64). In addition, TLRs may be important in the development of APCs. A study by Krutzik *et al*. (376) suggested that TLR 2, 4, and 5 activation may trigger the rapid differentiation of human monocytes into DC-SIGN$^+$ CD16$^+$ MΦ and CD1b$^+$ DC-SIGN$^-$ DC. In addition, it was previously demonstrated that MΦ pulsed in vitro with soluble protein in the absence of a TLR ligand, elicit a predominant T_H2 antibody response upon adoptive transfer (223, 306), whereas additional stimulation with a TLR ligand resulted in the induction of both T_H1 and T_H2 IgG isotypes, similar to DC pulsed with antigen in the absence of TLR stimulation (305).

Therefore, APCs activated by a soluble protein antigen (i.e toxins) may modulate a different humoral response compared to APC activated by an intact pathogen that contains multiple TLR ligands.

The ability of MΦ to elicit a humoral response

There have been many reports indicating that MΦ are able to stimulate already activated (effector) CD4+ T cells or hybridomas by acquiring Ag and presenting the peptides on their MHC class I or II molecules (330, 331, 340, 377-383). However the prevailing view is that not only are DC substantially better at priming naïve T cells than MF but that naïve CD4+ T cells require a higher degree of stimulation than effector CD4+ T cells. Early studies demonstrated that DC were at least 100-fold more potent at stimulating a primary mixed leukocyte reaction (MLR) than macrophages or B cells (294-296). Inaba *et al*. pulsed both MΦ and DC with protein Ag in vitro, injected the cells into mice, and T cells from lymph nodes were challenged with protein Ag in vitro. T cells from mice that were injected with protein-pulsed DC were able to be primed. However, little or no priming was seen if Ag-pulsed MΦ were injected (297). They also found that $CD4^+$ transgenic T cells proliferated in vitro in response to Ag after Ag-pulsed DC, but not MΦ or B cells, that were adoptively transferred into the transgenic mice (299). Croft *et al*. compared the ability of MΦ, B cells and DC to prime naïve $CD4^+$ transgenic T cells in vitro that recognize a peptide of cytochrome C. They found that all APC populations induced T cell proliferation and secretion of IL-2, but DC were better stimulators at low cell numbers (298). However, a study by Askew *et al*. suggested that IFN-γ and IL-12 may be a key component for the effective Ag presentation of MΦ to naïve T cells. The progeny of individual macrophage precursors from mouse spleen were examined for their ability to constitutively process and present native pigeon cytochrome c or peptide fragment of this antigen to naïve CD4+ T cells from mice transgenic for a

Vα11/Vβ3 TCR that recognizes an epitope in the antigen fragment. Askew *et al.* showed that 20% of splenic MΦ progeny that were pulsed with Ag were able to induce proliferation and IL-2 secretion by T cells in vitro. In addition adding rIFN-γ promoted all MΦ subcultures to Ag-presenting status, suggesting that the ability to elicit IFN-γ from $CD4^+$ T cells is a major factor in whether or not MΦ acquire the Ag-presenting phenotype (300).

There have been several imaging studies designed to visualize the APCs that interact with naïve T cells in situ. Ingulli et al. used fluorescently labeled transgenic CD4+ T cells specific for an OVA peptide to investigate APC-CD4+ T cell priming. This group found that not only did OVA pulsed APCs clustered around T cells but that the transgenic T cells that were activated by DCs proliferated and secreted IL-12 (384). In another set of experiments by this same group, a role for CD11b+ DC in the presentation of soluble OVA to CD4+ T cells was demonstrated (385). In these experiments, DO11.10 T cells were adoptively transferred into BALB/c hosts. Twenty-four hours later, the mice were inoculated subcutaneously with flourochrome-labeled OVA. When the draining lymph nodes were removed 18 hours later and analyzed by FACS, CD8+ DC, a subpopulation of B cells and MΦ contained low amounts of OVA, whereas a subpopulation of $CD11b^+$ DCs contained large amounts of OVA. This group went on to show that only $CD11b^+$ DCs sorted from soluble OVA-injected mice could stimulate DO11.10 T cells to proliferate in vitro. In another study by Stoll *et al.*, DCs were in vitro-pulsed with peptide Ag and subcutaneously transferred into naïve mice. Naïve $CD4^+$ TCR transgenic T cells were injected i.v. and lymph nodes were removed for four-dimensional confocal imaging of T cells and DCs. This group observed DC-T cell

binding and subsequent T cell proliferation (386). Norbury *et al*. infected mice with a vaccinia virus expressing enhanced green fluorescent protein. They observed clustering of TCR-Tg $CD8^+$ T cells around GFP-positive (infected) DC but not MΦ (387). However in this study T cell activation was inferred by DC-T cell clustering and actual stimulation of T cell by DC was not witnessed. In fact, DCs can cause T cell clustering, even in the absence of specific Ag (388).

Several in vivo studies using toxic liposomes to deplete mice of macrophages were initially interpreted to show that MΦ are able to prime naïve T cells (389-392). However, a major flaw in these studies is that immature DCs are highly phagocytic and are able to ingest, and be killed by the toxic liposomes. Jung *et al.* used Tg mice that express the receptor for diphtheria toxin (DT) under the control of the CD11c promoter. After injection with DT, cells expressing the CD11c receptor disappeared and the subsequent CD8+ T cell mediated immune response to several Ag was inhibited. From these findings Jung et al determined that DC are the key components in mounting a T cell dependent immune response. However, several caveats arise from this system. Monocytes that become tissue macrophages express CD11c on their surface (393). Also, when MΦ phagocytose dying DC they can be affected when the toxins enter the phagosome and are transferred to the cytosol (340). Therefore it is possible that macrophages were depleted along with DC after the addition of the diphtheria toxin. One study did find that naïve CD8+ T cells were able to be stimulated by MΦ and become effector T cells in vivo after adoptively transferring viral Ag pulsed MF into naïve mice (308). In summary, DCs can stimulate primary T cell responses and may be particularly

potent in doing so. However, the evidence that they are not the only cell that can prime responses, and evidence that MΦ lack this capability is relatively scarce.

Even more scarce are reports investigating if MΦ can induce a primary humoral response. In fact, to our knowledge, there has been only one report investigating the ability of Mϕ to induce a humoral response in a naïve animal. In this study splenic DC and peritoneal Mϕ were pulsed in vitro with soluble protein Ag and adoptively transferred into naïve mice. Both pulsed DC and Mϕ induced the synthesis of specific antibodies but with different isotype profiles. Ag-pulsed DC induced the production of IgG2a and IgG1 while pulsed MΦ induced IgG1 and IgE antibodies (223). However it was unclear whether the MΦ was playing an active role in this process or was either transferring Ag to another APC or in some way activating another APC to elicit the humoral response.

Previous work in our lab have shown that BMDC that were incubated with *S. pneumoniae* in vitro secreted both the pro-inflammatory cytokines IL-6, IL-12 and TNF-α and the anti-inflammatory cytokine IL-10. Additionally, in vitro pulsed and transferred BMDC elicited both protein and polysaccharide specific antibodies in naïve mice. The ability of BMDC to induce a humoral response was T cell dependent and required both BMDC MHC class II surface expression and viability (43). Hence, we were interested in the ability of MΦ to induce a humoral response in naïve animals. We demonstrate that both bone marrow derived macrophages (BMM) and peritoneal macrophages (PerM) secrete cytokines in response to the Pn. In addition Pn pulsed-BMM and PerM are able to induce a primary Ig response specific for both bacterial- derived protein (PspA) and polysaccharide (PPS14). Pulsed BMM also induced immunologic memory for PspA.

The requirement for active participation for MΦ induction of anti-protein Ig response to intact Pn is highlighted by the fact that Pn-pulsed paraformaldehyde-fixed BMM were unable to induce a protein specific-IgG response. In addition to a requirement for viable BMM, the protein specific-IgG responses induced by pulsed BMM also share a requirement for T cells. The parameters governing the anti-protein versus the anti-polysaccharide responses were different. Specifically the anti-protein response showed a complete dependence on viable MΦ, T cells and a requirement for expression of CD40 and MHC II on the MΦ. In contrast the anti-polysaccharide response was overall T cell independent, did not require live MΦ nor CD40 and MHC II expression on MΦ.

The observation that Ag-pulsed MΦ induce a strong humoral response in vivo was unexpected since these cells express low levels of class II molecules and have been shown to be poor activators of peptide specific naïve T cells in vitro and in vivo (299). However, one study indicated that adding rIFN-γ to culture promoted MΦ to Ag-presenting status, suggesting that the ability to elicit IFN-γ from CD4+ T cells is a major factor in whether or not MΦ acquire the Ag-presenting phenotype (300). Since there is evidence that MΦ-derived IL-12 elicits IFN-γ from naïve CD4+ T cells (394), it possible that the injected macrophages acquire the capacity to prime T cells following activation in vivo.

Cognate interactions activate various receptors on both the APC and T cells (395). Specifically, T cells are activated after peptide laden MHC bind to TCR and costimulatory molecules such as CD80 and CD86 on the APC bind to their specific receptors (i.e. CD28). Following TCR activation, T cell upregulation of CD40 ligand

occurs with subsequent activation of the APC through CD40-CD40 ligand binding. In this regard, the absolute requirement for BMM expression of CD40 and MHC II for induction of PspA-specific IgG strongly supports the notion that cognate MΦ-T cell interactions play a critical role in mediating the protein response.

However, the fact that Ag-pulsed MΦ were able to induce a polysaccharide-specific Ig response even in the absence of MΦ surface expression of MHC class II and CD40 along with the fact that there was no requirement for live MΦ or the presence of T cells suggests a different mechanism for the anti-polysaccharide vs anti-protein response. One explanation for this dichotomy could be the transfer of Ag from the MΦ to another APC. One APC candidate to acquire MΦ Ag is the DC. However studies have shown that MΦ might not be able to transfer Ag to DCs. Crowley *et al*. demonstrated that addition of Ag-pulsed Mϕ to unpulsed DC did not allow the DC to stimulate T cell clones, indicating that peptides were not transferred from MΦ to DC in vitro (301). Pancholi *et al.* demonstrated T cells were unable to be stimulated after incubation with MΦ and DC containing mycobacterial Ag (338). A more likely mechanism is MΦ-B cell Ag transfer. In fact an imaging study using two-photon microscopy showed that antigen-specific B cells were able to encounter and capture immune complexes on MΦ through their B cell antigen receptor (BCR) (336).

One of the surprising aspects of this study was the fact that Ag-pulsed and paraformaldehyde-fixed MΦ were able to induce a polysaccharide response. Theoretically, Ag-pulsed and fixed MΦ might still have some Ag or intact bacteria on their surface. It therefore could be possible for B cells to acquire MΦ surface Ag and induce a polysaccharide response. The dichotomy witnessed with the anti-protein vs anti-

polysaccharide response could be a matter of the quantity of Ag needed to initiate a response. In other words it might be that much less Ag is needed for a polysaccharide vs protein response. In fact there are several reports indicating that B cells can be stimulated to proliferate, undergo Ig secretion and class switching directly through multivalent crosslinking of anti-IgD-dextran, which mimics the repeating sugars of a polysaccharide, without the need of T cell (396, 397). Goeckeritz *et al.* demonstrated that after activation by multivalent crosslinking with dextran-conjugated anti-IgD, in vitro and subsequent incubation with ODN (both methylated and non-methylated), B cells were able to proliferate and secrete antibodies. (396). Boswell *et al.* showed that MΦ pulsed with the polysaccharide thymus-independent Ag TNP-Ficoll could activate B cells for antibody formation and that the B cells stimulated by pulsed Mϕ are of a distinct B cell subset ($Lyb5^+$) (344, 348). Therefore it might be possible for low concentrations of a multi-epitope-expressing microorganism in the context of mammalian (methylated) or microorganism (non-methylated) DNA to induce B cell proliferation and Ig secretion

There are still many unanswered questions regarding the role of MΦ in eliciting a primary humoral response. It is still not clear whether the amount of Pn phagocytosed by MΦ vs DC or the amount of cells adoptively transferred have an effect on the elicitation of a humoral response both in the quantity of antibodies produced and distribution of isotypes. It may be that DC are indeed more efficient than MΦ at initiating a humoral response when there are lower amounts of bacteria present. Similarly, DC may be more efficient than MΦ when there are less cells present at the site of infection. To investigate this question DC and MΦ could be incubated with various amounts Pn14: cells (i.e. 1:1 , 10:1, 50:1, 100:1…), followed by transfer of the cells into naïve mice. A similar

experiment could be performed incubating the MΦ and DC with the ratio of Pn14:cells used in this thesis (2000:1), but transferring different amounts of cells into the naïve mice (i.e. 10^6, 10^5, 10^4, 10^3…). I would predict that at lower amounts of bacteria DC would be more efficient at eliciting a humoral response in relation to MΦ.

As mentioned earlier, there is evidence IFN-γ from CD4+ T cells are a major factor in whether or not MΦ acquire the Ag-presenting phenotype (300), suggesting that injected MΦ acquire the capacity to prime T cells following activation in vivo. To test this hypothesis, MΦ and DC could be incubated with various amounts recombinant INF-γ and Pn14 in vitro and assayed for the upregulation of MHC class II, CD80, CD86, and CD40. IFN-γ could be added before Pn14 or after Pn14 to investigate if IFN-γ works by somehow preactivating the cells or is important after the phagocytosis of the bacterium. I would expect that incubation with IFN-γ and Pn14 would increase the upregulation of MHC class II, and maybe CD80, CD86, and CD40.

Another issue left unresolved is whether soluble antigen induces a different humoral response than an intact bacterium. MΦ and DC could be incubated with a purified polysaccharide antigen, a purified protein antigen, or a bacterium and adoptively transferred into naïve mice. On day 7, 14, and 21 mice would be bled with boosting with free bacterium, protein or polysaccharide on day 14. The serum samples then would be assayed for quantity of antibodies produced and isotype distribution. In fact a report indicated that MΦ and DC pulsed with a soluble protein and adoptively transferred into naïve mice differentially regulated the development of T lymphocytes. DC induced a T_H1 phenotype while MΦ induced a T_H2 phenotype (307). Therefore in light of this evidence I would predict that MΦ and DC that are pulsed with soluble antigen and transferred into

naïve mice would elicit different isotype profiles. I would also predict that both the amount of antibodies elicited and isotype profile would be different if MΦ and DC were pulsed with soluble antigen vs an intact bacterium.

Experiments could be performed investigating where the adoptively transferred Pn, or soluble antigen-MΦ and DC traffic in the spleen (i.e. whether they traffic to the T cell zone or directly to the B cell zone). This could be performed by first labeling the bacterium, purified protein, or purified polysaccharide with a fluorescent dye and incubate MΦ or DC with these fluorescently labeled Ags. Then adoptively transfer the MΦ or DC, take the spleen out on various time points and use fluorescent confocal microscopy to analyze DC and MΦ trafficking. I would expect soluble Ag pulsed DC and MΦ to migrate to different parts of the spleen relative to Pn14 pulsed- DC and MΦ.

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